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THE PRINCIPLES
OF
BACTERIOLOGY

A PRACTICAL MANUAL FOR STUDENTS
AND PHYSICIANS

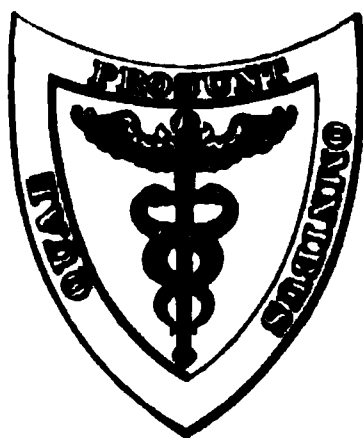
BY

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NINTH EDITION, THOROUGHLY REVISED

WITH 113 ILLUSTRATIONS, 28 OF WHICH ARE COLORED



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PREFACE TO NINTH EDITION.

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IN this, the ninth edition of the *Principles of Bacteriology*, a number of changes have been made, though they do not constitute a departure from the original objects of the work. It still remains a book for the beginner.

The order in which some of the matter was presented has been changed to what seems a more logical sequence. Several chapters have been fully revised and rewritten, and the section upon the physiological functions of bacteria has been greatly extended. New matter bearing upon hemolysis, complement-fixation, and the Ehrlich conception of the reactions of immunity has been introduced.

Such new principles and their applications as have been found worthy of serious consideration have been incorporated, and a large amount of old matter, important historically but not otherwise necessary, has been eliminated.

Nothing has been left undone to improve the book and bring it abreast of the very active subject. It is hoped that in its new form the work will continue to receive the flattering reception accorded to its preceding editions.

A. C. A.

UNIVERSITY OF PENNSYLVANIA, 1915.

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medicine. Though the contributions which have done most to place bacteriology on the footing of a science are those of recent years, still, during the earlier stages of its development, many observations were made which formed the foundation-work for much that was to follow. Before regularly beginning our studies, therefore, it may be of advantage to acquaint ourselves with the more prominent of those investigations.

Antony van Leeuwenhoek, the first to describe the bodies now recognized as bacteria, was born at Delft, in Holland, in 1632. He was not considered a man of liberal education, having been during his early years an apprentice to a linen-draper. During his apprenticeship he learned the art of lens-grinding, in which he became so proficient that he eventually perfected a simple lens by means of which he was enabled to see objects of much smaller dimensions than any hitherto seen with the best compound microscopes in existence at that date. At the time of his discoveries he was following the trade of linendraper in Amsterdam.

In 1675 he published the fact that he had succeeded in perfecting a lens by means of which he could detect in a drop of rain-water living, motile "animalcules" of the most minute dimensions—smaller than anything that had hitherto been seen. Encouraged by this discovery, he continued to examine various substances for the presence of what he considered animal life in its most minute form. He found in sea-water, in well-water, in the intestinal canal of frogs and birds, and in his own diarrheal evacuations, objects that differentiated themselves the one from the other, not only by their shape and size, but also by the peculiarity of motility which some of them were seen to possess. In the year 1683 he discovered in the tartar scraped from between

the teeth a form of micro-organism upon which he laid special stress. This observation he embodied in the form of a contribution to the Royal Society of London on September 14, 1683. This paper is of peculiar importance, not only because of the careful, objective nature of the description given of the bodies seen by him, but also for the illustrations which accompany it. From a perusal of the text and an inspection of the plates there remains little room for doubt that Leeuwenhoek saw with his primitive lens the bodies now recognized as bacteria.¹

Upon seeing these bodies he was apparently very much impressed, for he writes: "With the greatest astonishment I observed that everywhere throughout the material which I was examining were distributed animalcules of the most microscopic dimensions, which moved themselves about in a remarkably energetic way."

This discovery was shortly followed by others of an equally important nature. His field of observation appears to have increased rapidly, for after a time he speaks of bodies of much smaller dimensions than those at first described by him.

Throughout all of Leeuwenhoek's work there is a conspicuous absence of the speculative. His contributions are remarkable for their purely objective nature.

After the presence of these organisms in water, in the mouth, and in the intestinal evacuations was made known to the world, it is not surprising that they were immediately seized upon as the explanation of the origin of many obscure diseases. So universal became the belief in a causal relation between the "animalcules" and disease that it amounted

¹ See *Arcana Naturæ detecta ab ANTONIO VAN LEEUWENHOEK*; Delphis Batavorum, 1695.

almost to a germ-mania. It became the fashion to suspect the presence of these organisms in all forms and kinds of disease, simply because they had been demonstrated in the mouth, intestinal evacuations, and water.

Though nothing of value at the time had been done in the way of classification, and even less in separating and identifying the members of this large group, still the foremost men of the day did not hesitate to ascribe to them not only the property of producing pathological conditions, but some even went so far as to hold that variations in the symptoms of disease were the result of differences in the behavior of the micro-organisms in the tissues.

Marcus Antonius Plenciz, a physician of Vienna in 1762, declared himself a firm believer in the work of Leeuwenhoek, and based the doctrine which he taught upon the discoveries of the Dutch observer and upon observations of a confirmatory nature which he himself had made. The doctrine of Plenciz assumed a causal relation between the micro-organisms discovered and described by Leeuwenhoek and all infectious diseases. He maintained that the material of infection could be nothing else than a living substance, and endeavored on these grounds to explain the variations in the period of incubation of the different infectious diseases. He likewise believed the living contagium to be capable of multiplication within the body, and spoke of the possibility of its transmission through the air. He believed in the existence of a special germ for each disease, holding that just as from a given cereal only one kind of grain can grow, so by the special germ for each disease only that disease can be produced.

He found in all decomposing matters innumerable minute "animalculæ," and was so firmly convinced of their etio-

logical relation to the process that he formulated the law: that decomposition can only take place when the decomposable material becomes coated with a layer of the organisms, and can proceed only when they increase and multiply.

However convincing the arguments of Plenciz may appear, they seem to have been lost sight of in the course of subsequent events, and by a few were even regarded as the productions of an unbalanced mind. For example, as late as 1820 we find Ozanam expressing himself on the subject as follows: "Many authors have written concerning the animal nature of the contagion of disease; many have indeed assumed it to be developed from animal substances, and that it is itself animal and possesses the property of life; I shall not waste time in effort to refute these absurd hypotheses."

Similar expressions of opinion were heard from many other investigators of the time, all tending in the same direction, all doubting the possibility of these microscopic creatures belonging to the world of living things.

It was not until between the fourth and fifth decades of the nineteenth century that by the fortunate coincidence of a number of important discoveries the true relation of the lower organisms to infectious diseases was scientifically pointed out. With the fundamental investigations of Pasteur upon the souring and putrefaction of beer and wine; with the discovery by Pollender and Davaine of the presence of rod-shaped organisms in the blood of animals dead of splenic fever, and with the progress of knowledge upon the parasitic nature of certain diseases of plants, the old question of "*contagium animatum*" again began to receive attention. It was taken up by Henle, and it was he who first logically taught this doctrine of infection.

The main point, however, that had occupied the attention of scientific men from time to time for a period of about two hundred years subsequent to Leeuwenhoek's discoveries was the origin of the "animalcules." Do they generate spontaneously, or are they the descendants of pre-existing creatures of the same kind? was the all-important question. Among the earlier participants in this discussion were many of the most distinguished men of the day.

In 1749 Needham, who held firmly to the opinion that the bodies which were attracting such general attention developed spontaneously as the result of vegetative changes in the substances in which they were found, attempted to demonstrate by experiment his reasons for holding this view. He maintained that the bacteria which appeared about a grain of barley germinating in a carefully covered watch-crystal of water were the result of changes going on in the barley-grain itself, incidental to its germination.

Spallanzani, in 1769, drew attention to the laxity of Needham's experimental methods, and demonstrated that if infusions of decomposable vegetable matter be placed in flasks, which, after being hermetically sealed, were heated for a time in boiling water, no living organisms would be detected in them, nor would decomposition appear in the infusions so treated. The objection raised by Treviranus, viz., that the high temperature to which the infusions had been subjected had so altered them and the air about them that the conditions favorable to spontaneous generation no longer existed, was promptly met by Spallanzani when he gently tapped one of the flasks that had been boiled against a hard object until a minute crack was produced; invariably organisms and decomposition appeared in the flask thus treated.

From the time of the experiments of Spallanzani until as late as 1836 but little advance was made in the elucidation of this, at that time, obscure problem.

In 1836 Schulze attracted attention to the subject by the convincing nature of his investigations. He showed that if the air which gained access to boiled infusions be robbed of its living organisms by first passing it through strong acid or alkaline solutions no decomposition occurred, and living organisms could not be detected in the infusions. Following quickly upon this contribution came Schwann, in 1837, and somewhat later (1854) Schröder and Dusch, with similar results obtained by somewhat different means. Schwann deprived the air which passed to his infusions of its living particles by conducting it through highly heated tubes; whereas Schröder and Dusch, by means of cotton-wool interposed between the boiled infusions and the outside air, robbed the air passing to the infusions of its organisms by the simple process of filtration. In 1860 Hoffmann and in 1861 Chevreul and Pasteur demonstrated that the precautions taken by preceding investigators for rendering the air which entered these flasks free from bacteria were not necessary; that all that was required to prevent the access of bacteria to the infusions in the flasks was to draw out the neck of the flask into a fine tube, bend it down along the side of the flask, and then bend it up again a few centimeters from its extremity, and leave the mouth open. The infusion was then to be boiled in the flask thus prepared and the mouth of the tube left open. The organisms which now fell into the open end of the tube were arrested by the drop of water of condensation which collected at its lowest angle, and none could enter the flask.

While, from our modern standpoint, the results of these

investigations seem to be of a most convincing nature, yet there were many at the time who required additional proof that "spontaneous generation" was not the explanation for the mysterious appearance of these minute living creatures. The majority, if not all, of such doubts were subsequently dissipated through the well-known investigations of Tyndall upon the floating matters of the air. In these studies he demonstrated by numerous ingenious and instructive experiments that the presence of living organisms in decomposing fluids was always to be explained either by the pre-existence of similar living forms in the infusion or upon the walls of the vessel containing it, or by the infusion having been exposed to air which had not been deprived of its viable organisms.

Throughout all the work bearing upon this subject, from the time of Spallanzani to that of Tyndall, certain irregularities were constantly appearing. It was found that particular substances required to be heated for a much longer time than was needed to render other substances free from living organisms, and even after heating under the most careful precautions decomposition would occasionally occur.

In 1762 Bonnet, who was deeply interested in this subject, suggested, in reference to the results obtained by Needham, the possibility of the existence of "germs or their eggs," which had the power to resist the temperature to which some of the infusions employed in Needham's experiments had been subjected.

More than a hundred years after Bonnet had indulged in this pure speculation it became the happy privilege of Ferdinand Cohn, of Breslau, to demonstrate its accuracy and importance.

Cohn repeated the foregoing experiments with like results.

He concluded that the irregularities could only be due to either the existence of more resistant species of bacteria or to more resistant stages into which certain bacteria have the property of passing. He demonstrated that some of the rod-shaped organisms possess the power of passing into a resting- or spore-stage in the course of their life-cycle, analogous to the seeding stage of higher plants, and when in this stage they are much less susceptible to the deleterious action of high temperatures than when they are growing as normal vegetative forms. With the discovery of these more resistant spores the doctrine of spontaneous generation received its death-blow. It was no longer difficult to explain the inconsistencies in the results of former investigations, nor was it any longer to be doubted that putrefaction and fermentation were the result of bacterial life and not the cause of it, and that these bacteria were the offspring of pre-existing similar forms. In other words, the law of Harvey, *Omne vivum ex ovo*, or its modification, *Omne vivum ex vivo*, was shown to apply not only to the more highly organized members of the animal and vegetable kingdoms, but to the most microscopic, unicellular creatures as well.

The establishment of this point gave an impetus to further investigations, and as the all-important question was that concerning the relation of the microscopic organisms to disease, attention naturally turned into this channel of study. Even before the hypothesis of spontaneous generation had received its final refutation a number of observations of a most important nature had been made by investigators who had long since ceased to consider spontaneous generation as a tenable explanation of the origin of the microscopic living particles.

In the main, these studies had been conducted upon wounds and the infections to which they are liable; in fact, the evolution of our knowledge of bacteriology to its present development is so intimately associated with this particular line of investigation that a few historical facts in connection with it may not be without interest.

The observations of Rindfleisch, in 1866, in which he describes the presence of small, pin-head points in the myocardium and general musculature of individuals that had died as a result of infected wounds, represent, probably, the first reliable contribution to this subject. He studied the tissue-changes round about these points up to the stage of miliary abscess-formation. He refers to the organisms as "vibrios." Almost simultaneously von Recklinghausen and Waldeyer described similar changes that they had observed in pyemia and, occasionally, secondary to typhoid fever. Von Recklinghausen believed the granules seen in the abscess-points to be micrococci and not tissue-detritus, and gave as the reason that they were regular in size and shape, and gave specific reactions with particular straining-fluids. Birch-Hirschfeld was able to trace bacteria found in the blood and organs to the wound as the point of entrance, and believed both the local and the constitutional conditions to stand in direct ratio to the number of spherical bacteria present in the wound. He observed also that as the organisms increased in number they could often be found within the bodies of pus-corpuscles. His studies of pyemia led him to the important conclusion that in this condition micro-organisms were *always* present in the blood.

Of immense importance to the subject were the investigations of Klebs, made at the Military Hospital at Karlsruhe

in 1870-'71. He not only saw, as others before him had seen, that bacteria were present in diseases following infection of wounds, but described the manner in which the organisms had gained entrance from the point of injury to the internal organs and blood. He expressed the opinion that the spherical and rod-shaped bodies which he saw in the secretions of wounds were closely allied, and he gave to them the designation "microsporon septicum." He believed that the organisms gained access to the tissues round about the point of injury both by the aid of the wandering leukocytes and by being forced through the connective-tissue lymph-spaces by the mechanical pressure of muscular contraction.

On erysipelatous inflammations secondary to injury important investigations were also being made, Wilde, Orth, von Recklinghausen, Lukomsky, Billroth, Ehrlich, Fehleisen, and others agreeing that in these conditions micro-organisms could always be detected in the lymph-channels of the subcutaneous tissues; and through the work of Oertel, Nassiloff, Classen, Letzerich, Klebs, and Eberth the constant presence of bacteria in the diphtheritic deposits at times seen on open wounds was established.

Simple and natural as all this may seem to us now, the stage to which the subject had developed when these observations were recorded did not admit of their meeting with unconditional acceptance. The only strong argument in favor of the etiological relation of the organisms that had been seen to the diseases with which they were associated was the constancy of this association. No efforts had been made to isolate them, and few or none to reproduce the pathological conditions by inoculation. Moreover, not a small number of investigators were skeptical as to the

importance of these observations; many claimed that micro-organisms were normally present in the blood and tissues of the body; and some even urged that the organisms seen in diseased conditions were the result rather than the cause of the maladies. It is hardly necessary to do more than say that both of these views were purely speculative, and have never had a single reliable experimental argument in their favor. Billroth and Tiegel, who held to the former opinion, did endeavor to prove their position through experimental means; but the methods employed by them were of such an untrustworthy nature that the fallacy of deductions drawn from them was very quickly made manifest by subsequent investigators. Their method for demonstrating the presence of micro-organisms in normal tissues was to remove bits of organs from the healthy animal body with heated instruments and drop them into hot melted paraffin. They held that all living organisms on the surface of the tissues would be destroyed by the high temperature, and that if decomposition should subsequently occur it would prove that it was the result of the growth of bacteria in the depths of the tissues to which the heat had not penetrated. Decomposition did usually set in, and they accepted this as proof of the accuracy of their view. Attention was, however, shortly called to the fact that in cooling there was contraction of paraffin, resulting usually in the production of small rents and cracks in which dust, and bacteria lodged upon it, could accumulate and finally gain access to the tissues, with the occurrence of decomposition as a consequence. Their results were thus explained after a manner analogous to that employed by Spallanzani, in 1769, in demonstrating to Treviranus the fallacy of the opinion held by him and the accuracy of his own views, viz., that it was always through

the access of organisms from without that decomposition primarily originated. (See page 22.)

Under careful precautions, to which no objection could be raised, the experiments of Billroth and Tiegel were repeated by Pasteur, Burdon-Sanderson, and Klebs, but with failure in every instance to demonstrate the presence of bacteria in the healthy living tissues.

The fundamental researches of Koch (1881) upon pathogenic bacteria and their relation to the infectious diseases of animals differed from those of preceding investigators in many important respects. The scientific methods of analysis with which each and every obscure problem was met as it arose served at once to distinguish him as a pioneer in this hitherto but imperfectly cultivated domain. The outcome of these investigations was the establishment of a foundation upon which bacteriology of the future was to rest. He, for the first time, demonstrated that distinct varieties of infection, as evidenced by anatomical changes, are due in many cases to the activities of specific micro-organisms, and that by proper methods it is possible to isolate these organisms in pure culture, to cultivate them indefinitely under artificial conditions, to reproduce the lesions by inoculation of these pure cultures into susceptible animals, and to continue the disease at will by continuous inoculation from an infected to a healthy animal. By the methods that he employed he demonstrated a series of separate and distinct diseases that can be produced in mice and rabbits by the injection of putrid substances into their tissues. The disease known as septicemia of mice; likewise a disease characterized by progressive abscess-formation, and pyemia and septicemia of rabbits, were among the affections first produced by him in this way. It was in the

course of this work that the Abbe system of substage condensing apparatus was first used in bacteriology; that the aniline dyes suggested by Weigert were brought into general use; that the isolation and cultivation of bacteria in pure culture on solid media were shown to be possible; and that animals were employed as a means of obtaining from mixtures pure cultures of pathogenic bacteria.

With the bounteous harvest of original and important suggestions that was reaped from Koch's classical series of investigations bacteriology reached an epoch in its development, and at this period modern bacteriology may justly be said to have had its birth.

NOTE.—I have presented only the most prominent investigations that will serve to indicate the lines along which the subject has developed. For a more detailed account of the historical development of the work the reader is referred to Löffler's instructive and entertaining *Vorlesungen über die geschichtliche Entwicklung der Lehre von den Bakterien*, upon which I have drawn freely in preparing the foregoing sketch.

CHAPTER I.

Definition of Bacteria—Differences Between Parasites and Saprophytes
—Their Place in Nature—Bacterial Enzymes—Products of Bacteria
—Nutrition of Bacteria—Their Relation to Oxygen—Influence of
Temperature Upon Their Growth—Chemotaxis.

BACTERIA (more properly bacteriaceæ or schizomycetes) were regarded by the older writers as infusoria. This was because of their capacity for developing in infusions, their property of spore-formation, their resistance to drying, their power of independent motion, and the absence of chlorophyl from their tissues. In the modern conception, however, this classification is untenable, and bacteria, by virtue of their distinguishing peculiarities, are now treated as a group by themselves that may briefly be defined as comprising microscopic, unicellular, vegetable organisms that multiply by the process of transverse division.

Inasmuch as bacteria are not possessed of chlorophyl, their metabolic processes are fundamentally different from those of the higher plants in which it is present. They cannot, as in the case of the green plants, obtain carbon and nitrogen from such simple bodies as carbon dioxide and ammonia, but are forced to secure these essential elements from organic matter as such. This power to decompose and assimilate organic matters is signally different in different species of bacteria, and, singular to say, there is a small group (to be described later) from which this function is apparently absent, in spite of the fact that no compensatory chlorophyl is discernible in their tissues.

SAPROPHYTES AND PARASITES. — In the case of certain bacteria, in fact, the majority, the source of food-supply must of necessity be dead organic matters of either animal or vegetable origin. They cannot exist in the presence of living tissues. To the members of this group the designation *saprophytic* or *metatrophic* (A. Fischer) is given. To that group that can exist only upon *living* organic matters, and herein belong many (not all) of the disease-producing bacteria, the appellation *parasitic* or *paratrophic* (A. Fischer) is applied; while for the few species that either do not require organic matters, or do not, so far as is known, have the faculty of decomposing and assimilating proteid stuffs at all, the name *prototrophic* is suggested by Fischer. In the strict sense of the word, a parasite can exist only in the body of a living host, and a saprophyte only upon lifeless organic matters, and such *obligate* parasites and saprophytes are known, but in the majority of cases such nutritive conditions are not obligatory, many of both parasites and saprophytes having the power to adapt themselves to conditions other than those for which they are by nature best fitted. For instance, certain species that exhibit their most important properties under conditions of parasitism may, nevertheless, lead a saprophytic existence when circumstances demand it, and, on the other hand, particular species usually saprophytic by nature may find conditions favorable to their development in a living host. To such adaptable species the designation "facultative" is given, and, when employed, signifies that the species in question has the faculty of adapting itself to environments other than those in which it is usually encountered. In this sense all of the disease-producing bacteria that can be cultivated artificially are manifestly facultative saprophytes.

The life-processes of bacteria are so rapid, complex, and energetic that they result in the most profound alterations in the structure and composition of the materials in and upon which they are developing.

Disintegrations and decompositions result from the activities of the saprophytic bacteria; while the changes brought about in the tissues of their living host by the purely *parasitic* forms find expression in disease-processes not infrequently leading to complete death.

THEIR PLACE IN NATURE.—The rôle played in nature by the saprophytes is a very important one. Through their functional activities the highly complicated tissues of dead animals and vegetables are resolved into the simpler compounds, carbonic acid, water, and ammonia, in which form they may be taken up and appropriated as nourishment by the more highly organized members of the vegetable kingdom. It is through this ultimate production of carbonic acid, ammonia, and water by bacteria, as end-products in the processes of decomposition and fermentation of dead animal and vegetable tissues, that the demands of growing vegetation for these compounds are supplied.

The more highly organized chlorophyl plants do not possess the power of obtaining their carbon and nitrogen from such complicated organic substances as serve for the nutrition of bacteria, and as the production of the simpler compounds, carbon dioxide and ammonia, by the animal world is not sufficient to meet the demands of the chlorophyl plants, the importance of the part played by bacteria in making up this deficit is obvious and cannot be overestimated. Were it not for the activity of these microscopic living creatures all life upon the surface of the earth would cease. Deprive higher vegetation of the carbon and nitrogen

supplied to it as a result of bacterial activity, and its development comes rapidly to an end; rob the animal kingdom of the food-stuffs supplied to it by the vegetable world, and life is no longer possible. It is plain, therefore, that in this cycle of life phenomenon the saprophytes, which represent the large majority of all bacteria, must be looked upon in the light of benefactors, without which existence would be impossible.

With the parasites, on the other hand, the conditions are far from analagous. Through their metabolic activities there is constantly a loss, rather than a gain, to both the animal and vegetable kingdoms. Their host must always be a living body in which exist conditions favorable to their development, and from which they appropriate substances that are necessary to the health and life of the organism on which they are preying; at the same time they elaborate substances as products of their nutrition that are directly poisonous to the tissues in which they are growing.

In their relations to terrestrial life, therefore, the positions occupied by the two functionally different groups, the saprophytes on the one hand, and the parasites on the other, are diametrically opposed.

SPECIFIC FUNCTIONS OF SAPROPHYTIC BACTERIA.

Appropriate investigation of the saprophytic group of bacteria has shed important light upon certain specific characteristics with which many of the species are endowed. We know that numerous common phenomena are the results of their activities. The souring of milk, the ripening of cheese; certain of the fermentations resulting in the formation of various acids of the fatty series; the elaboration of

other aromatic bodies of organic character and origin; the spoiling of wine; the disintegrations incidental to the manufacture of hemp products; the old method of making indigo; the natural and artificial methods for the destruction of the organic waste encountered in polluted waters and sewage and the transformations of dead organic matter in the soil are all illustrations of these well-known phenomena. In a number of commercial lines constant use is made of these bacterial activities. This is conspicuously seen in the manufacture of butter and cheese where the excellence of the products is due to the peculiar flavors caused by bacterial growth in the raw materials. Before synthetic methods became so generally in use bacterial activities were largely employed in the manufacture of the organic acids.

In addition to the foregoing a number of saprophytes have the specific property of producing beautiful pigments, red, yellow, orange, pink, violet, green, etc. This group of "*chromogens*" as they are called have doubtless other functions in the great laboratory of nature, the soil, where they are commonly found, but color production is the most obvious.

Another group—the "*photogens*" or photogenic species have the remarkable ability to produce luminosity in the substances in or on which they exist. It is to the activity of this group that the phosphorescence sometimes seen in decayed wood, in rotten fish and other flesh is attributable. How it is done is a mystery, just as is the means by which the fire-fly and the glow-worm emit their tiny sparks of light.

Another group have as the end products of their activities those evil smelling bodies by which putrefaction is characterized, these are the so-called "*saprogenic*" species.

Others have as their most interesting functions the power

to carry hydrogen sulphide to higher sulphur compounds, the so-called "*thiogenic*" species.

Those saprophytes that are concerned in such well-known fermentations as result in the production of the various acids of the fatty series are known as "zymogens."

But of all the so-called saprophytic group none are more interesting and none by any means so important as those concerned in the various transformations through which nitrogen passes in being prepared as food for higher vegetation. This group, or rather those groups, for there are apparently several operating on nitrogen and its compounds in various ways, are known as the "*nitrifying*" and the "*denitrifying*" and the "*nitrogen fixing*" bacteria.

NITRIFYING BACTERIA.—They carry ammonia, resulting from the decomposition of dead animals and plants; by a process of oxidation first to nitrous acid, then by further oxidation the nitrous acid is carried to nitric acid. These two steps in the process are taken by two totally distinct groups of bacteria of a most interesting nature. The function of one group is strictly limited to the nitrite process; that of the other to the nitrate; the latter taking up the work at the point where the former leaves it. The former cannot carry its operation beyond the nitrite point, nor can the latter begin with ammonia and carry it to complete nitrification. A most singular peculiarity of this group is the inability to develop on the nutrient media commonly used for the cultivation of bacteria. Organic matter as such seems to be unfavorable to their viability. To grow them one is obliged to use a silicate jelly, a sort of water glass of about the consistency of ordinary gelatin, to which are added certain salts that these particular species are able to decompose in order to secure the elements necessary

to provide energy. In so far as life upon the earth's surface is concerned the nitrification going on in the soil as a result of the activities of this group is one of the most important phenomena in operation. It is to a large extent responsible for supplying higher vegetation with nitrogen in a form available for food.

Denitrification, i. e., the reverse of nitrification, the reduction of nitrates and nitrites to ammonia is a function peculiar to many bacteria, particularly many species found in the soil. Often it does not appear to be a specific function and is frequently accomplished under conditions where organic matter is present and is utilized. In many cases the denitrification seems to be less a phenomenon due to the specific activities of the bacteria themselves than to the reducing action of the products of their growth. In the case of the few species that have been called "true denitrifiers," the reduction appears to be due to the respiratory demands of those species for oxygen; this robbing of the oxides of nitrogen of their oxygen by the bacteria resulting, manifestly, in reduction.

Nitrogen Fixation.—Another phenomenon having to do with nitrogen, and resulting from the activity of saprophytes, is the so-called "nitrogen fixation" by bacteria. For many years we were taught that the nitrogen of the air, constituting about 80 per cent. of the entire atmosphere, was of no biological significance and was put there by nature merely to dilute the excessively active oxygen to a point compatible with respiration by man and animals. This extraordinary conception was always looked upon with suspicion by thoughtful students. It was not, however, until about 1886 that the real significance of atmospheric nitrogen was made clear. Hellrigel and Wilfarth at that time demonstrated

that the nodules found on the roots of the leguminous plants (clover, peas, beans, etc.), might properly be regarded as communities of bacteria which were beneficently co-operating with the plants in the performance of their fundamental life processes, *i. e.*, they were in "symbiotic" relationship. The result of this co-operation they showed to be the power of the legumens to fix and store the free atmospheric nitrogen. When one realizes how inexhaustible is the supply of free atmospheric nitrogen it is difficult to exaggerate the importance of this function. It also sheds interesting light upon certain practices of the agriculturalist that have been in empirical operation since the cultivation of the soil began. It has always been known that the rotation of crops is essential to the successful tillage of the soil and we find that in such rotation one or another of the legumens was always used. The reason is evident, they do not impoverish, but though their ability to fix nitrogen through the aid of the "nodule bacteria" on their roots, they actually enrich the soil.

From the foregoing it is obvious that the expression "nature's laboratory" is properly applied to the soil. It is here that all the saprophytes are sooner or later found. Among her various analytic and synthetic performances nature concerns herself with none so important to life as those having to do with the several transformations of nitrogen to which allusion has just been made.

SPECIFIC FUNCTIONS OF THE PARASITIC BACTERIA.

As already intimated the parasitic bacteria are not characterized by such beneficent activities as are possessed by the saprophytic group; they exist at the expense of living

hosts and usually excite detrimental changes in those hosts. It is to the parasitic group that the pathogenic or disease-exciting bacteria belong.

Strictly speaking none of the pathogenic bacteria with which we are acquainted are obligate parasites, that is, none of them grow and multiply only in the body of a living host; for all have been cultivated under artificial conditions on dead, nutrient cultural materials. They are nevertheless properly classified as parasites for it is only under conditions of parasitism that they exhibit those activities that make them the objects of special interest.

When circumstances admit of the various members of this group getting access to the living hosts in which they find conditions favorable to their growth and multiplication there results the state known as "disease." In some cases the disease is local, *i. e.*, it involves only the tissues in the immediate vicinity of the invading bacteria; in others it is general; involves the entire body and eventuates in the death of the host.

As we study the peculiarities of the disease-producing bacteria more closely we find that in inducing disease they do not all operate in the same way, though the ultimate forces used by them in the destruction of living tissue are throughout analogous, *i. e.*, they are poisons.

In some cases the parasite finds the circulating fluids of the host the most favorable place for its growth and development. Under such circumstances it is not uncommon for the blood and lymph vessels of an infected animal to be almost filled with the parasites within a short time after the invasion. To such a state the designation "septicemia" is given, that is, there is a septic condition of the blood, "blood poisoning" as it is commonly called.

In other instances a parasitic species may manifest its activities in a very insignificant way, insofar as the welfare of the host is concerned. The causation of simple boils, pimples and unimportant local inflammations serves to illustrate this. In other examples we find the activities of the parasite more or less confined to special vital organs of the body, the restriction, practically speaking, of the cholera and dysentery germs to the mucosa of the intestinal canal; of the gonorrheal germ to the mucous surfaces of the genito-urinary tract; of the typhoid bacillus to the lymphatic structures of the abdominal cavity may serve as illustrations.

Again we know of parasitic species that do not disseminate beyond their portal of entry. They grow at that point and manufacture deadly poisons which are disseminated throughout the body by way of the circulating fluids. The germs of diphtheria and of tetanus are striking illustrations of this type of parasite.

In practically all cases fever is an accompaniment of the activities of the parasitic bacteria in the body though in certain particular instances an initial rise in temperature may be followed by marked depressions of it, due to the action of the poisons elaborated by the bacteria.

In considering the activities of the parasitic group of bacteria we encounter at the beginning one factor in particular with which we are not called upon to reckon in speaking of the saprophytic group. The saprophytes work upon inert, dead matter; the parasites on active, living matter, all of which is by nature endowed with some degree of resistance to the inroads and activities of invading parasites. It is through this "vital resistance" possessed by the living host that many of the irregularities seen in the opera-

tion of the parasitic group may be explained. It is indeed so active in certain individual cases as to give to its possessor almost complete immunity from particular types of parasitic invasion.

To illustrate: Diphtheria is caused by a well-known parasite. It has many interesting properties but the most significant of its physiological activities is its power to elaborate a poison that causes the group of symptoms and tissue changes which we know as diphtheria. No other organism has this property. This same diphtheria bacillus may invade one person and cause his death, while in another the results of its activities may be comparatively trifling. Similar extremes of variation are constantly seen in the case of all the known infective diseases. It is to be explained in but one way "the soil," *i. e.*, the living host, in which the disease exciting "seed," the germ, finds itself is not that which is best suited to its active growth, or in other words one individual possess higher natural powers of resistance than does another, and in a large group of individuals such differences in the degree of resistance are marked. We see nothing like this in the action of saprophytes upon dead matter. It is true we see their growth restrained at times. In some cases such restraint is exercised by other species the products of whose growth are antagonistic; and in a number of cases the growth of saprophytes is often for a time checked by the accumulated products of their own activities. For instance the growth of those saprophytes concerned in acid fermentations comes very quickly to an end unless special provisions be made to neutralize and fix the acids as fast as they are manufactured, for no bacteria develop indefinitely in the presence of free acids. This is, however, a very different kind of inhibition from that

exercised by living tissues in repelling the invasion of parasites.

In the foregoing brief sketch of the manifold transformation resulting from bacterial activity there is no discussion of the mechanism through which such changes are wrought. In the case of the saprophytes the various analyses and syntheses that accompany their growth are in general believed to be manifestations of fermentations; while the activities of the parasites in producing disease are referred to poisons elaborated by these that have a destructive action upon the tissues in which the parasites are operating. In the following paragraphs an effort will be made to elucidate this phase of the subject.

FERMENTS, ENZYMES, TOXINS, BACTERIAL PROTEINS, AND PTOMAINS.

There is perhaps no department of either biology or physics that relates to more important phenomena, more widespread phenomena or more inexplicable phenomena than that having to do with fermentation and the agencies that cause it.

The phenomenon has attracted the attention of the ablest investigators for years and we are scarcely nearer to an understanding of its intimate nature today than we were at the beginning.

In its older sense, the word fermentation related to all reactions that are accompanied by the evolution of gas and, indeed, it is probable that the word originated with the word *fervere*, meaning to seethe, to boil, to bubble. In its modern usage, however, the word comprehends many reactions, believed to be caused by ferments, but during which no

gas as such is evolved: The fermentation best and longest known to man is that through which sugar is converted into alcohol, seen in the making of wine from grapes. In so far as bacteria are concerned we are aware of a multiplicity of reactions which are believed to be manifestations of fermentation, though opinion on these points is far from being in agreement. However, as ferments have never been isolated in a pure state and as the real nature of their activities cannot with the present means at our disposal, be finally determined, there is as much justification for regarding such reactions as excited by ferments as not. We shall therefore assume that both the normal metabolism of the bacterial cell and its peculiar power to excite specific reactions in various substances are made possible through the agency of ferments. In some cases such ferments are firmly bound up as integral parts of the cell protoplasm. To such cells with their peculiar ferments the term "organized ferments" is often applied. The common yeast cell serves as an example. In other cases the cells throw off in the course of their living activities, as by-products so to speak, bodies which, when completely separated from the cells by which they were formed, are still capable of bringing about fermentation reactions when mixed with appropriate substances, without themselves undergoing any demonstrable change. These are denominated "unorganized ferments" or "enzymes."

In the case of the disease-producing bacteria we have an analogous state of affairs. We find that the tissue changes characterizing disease are due to poisons elaborated by the living pathogens. These poisons are generically known as toxins, and it is possible, though not certain, that in causing disease their activities may be in some instances likened to

those of the enzymes of the non-disease producing group, while in others this is not the case. In the case of certain pathogens, as with the yeasts and certain saprophytic bacteria, these toxins—poisons—are so bound up with the protoplasmic bodies of the bacteria that they become effective as poisons only on the disintegration of the cells containing them; these are the “endotoxins.” In other instances the poisons are diffused through the surrounding medium in which the bacteria are growing and may readily be separated from the cells forming them by the simple process of filtration. These are the free or “true toxins.”

At one time there was believed to be an essential difference between the “organized” and “unorganized” ferments, but when in 1897 E. Buchner expressed the active ferment from the yeast cell, and demonstrated that this active principle, “zymase,” without the aid of the living cell, is capable of transforming sugar into alcohol, just as is done by the intact living yeast cells, it became manifest that the old distinction between “organized” and “unorganized” ferments is after all not important. The “enzyme” is the active agent and in so far as the result is concerned it matters not if it be tied up in the body of a cell or diffused freely in the medium surrounding the cell.

The same may be said with regard to the analogous “endotoxins” and “toxins” elaborated by the pathogenic species, though it must not be assumed that the toxins act in the same way as do the ferments or enzymes. Such knowledge as we have of the mechanism of certain toxic activities justifies the statement that the poisons of some pathogenic bacteria enter into a destructive combination with body cells for which they have a specific affinity and that there and then their activity ceases; the result being

that the physiological activities of both the poison and the cells are destroyed. Not so with the enzymes; they are characterized by the ability to bring about profound alterations in the substances on which they are acting without they themselves being appreciably altered or diminished in quantity; just as is seen with many of the inorganic catalysers which, after having induced the most profound and important reactions in the substances surrounding them, are found at the end to have undergone no loss in amount and to be of identically the same composition as at the beginning. As to the way in which enzymes act nothing definite can be said. The problem has for years engaged the attention of many competent investigators but up to the present no conclusion has been reached. That they differ in nature and mode of operation the one from the other seems certain; the results of their activities are manifestly different.

Neither enzymes nor toxins have ever been isolated in a pure state. Both are assumed to be amorphous matters of a protein nature and all are recognized by that which they do; *i. e.*, by the reactions which they originate. They are characterized for their instability, particularly is this the case with the enzymes. All have many of the essential characteristics of living matter; they are destroyed by heat, varying in amount and mode of application. The same chemicals that are hurtful to living cells are likewise, in the main, destructive of enzymes and toxins; they are soluble (or appear to be) in water, dilute acids, alkalies and neutral salines; they are to a slight extent dialyzable; some are precipitated from their solutions by alcohol readily, others less so; they may be thrown down from their solutions by mechanically enmeshing them with certain inorganic

precipitates. Their powers of fermentation (enzymes) and of intoxication (toxins) are apparently specific.

The enzymes of bacterial origin with which we are best acquainted may be defined as amorphous constituents of living protoplasm that are able through catalytic activity to split up complex organic substances into simpler, more soluble and diffusible combinations. They may be classified as proteolytic, diastatic, inverting, coagulating, sugar splitting, fat splitting, etc. It is important to note that such enzymes may and do originate in both the animal and vegetable world. Those obtained from bacteria are, in so far as it is possible to say, identical with those found in the cells of animals.

The *proteolytic* or albumin-dissolving enzymes are formed by a great many bacteria. The most familiar indications of the formation of a proteolytic enzyme are seen in the liquefaction of gelatin, in the digestion of coagulated blood-serum, and of casein. Most frequently the proteolytic enzyme is allied to trypsin, since the liquefaction, hydrolysis or digestion induced by it proceeds only under an alkaline reaction.¹ Some bacteria, however, produce a proteolytic enzyme analogous to pepsin, and this enzyme is active under an acid reaction. The proteolytic enzymes of different bacteria vary considerably with regard to their resistance to heat, some being destroyed in a few minutes when heated to 60° or 70° C., while others may be exposed to 100° C. for a short time without suffering marked deterioration.² The proteolytic enzymes also differ in respect to their susceptibility to the action of acids and other chemicals.

The formation of proteolytic enzymes is one of the func-

¹ See Abbott and Gildersleeve, Journ. of Med. Research, 1903, vol. v.

² Loc. cit.

tions of bacteria that is easily disturbed by external conditions, for instance, long-continued cultivation on media in which the exercise of this function is not required may lead to its marked deterioration, while prolonged cultivation under conditions demanding it may result in its accentuation.

The addition of carbohydrates and of glycerine to culture-media interferes with production of the proteolytic enzyme by many species of bacteria, as shown by Auerbach.¹

Diastatic enzymes convert starch into sugar. This function is best studied on media containing starch, as potato infusion or solutions of starch. By appropriate tests the intermediate steps in the conversion of the starch into sugar may be traced by testing a portion of the culture-medium from time to time. Fermi² found this function in a large number of bacteria studied, especially in organisms of the subtilis group and in the microspira of the cholera group.

Inverting enzymes convert saccharose into dextrose and levulose. These enzymes are produced by comparatively few bacteria. Fermi found this function manifested by bacillus megatherium, pseudomonas fluorescens, bacillus vulgaris, microspira comma, microspira Metchnikovi, and others.

Coagulating enzymes are those which coagulate milk. Rennet may be taken as the typical form. This alteration is quite common in association with an acid reaction, but in such instances it is not always certain that the coagulation has not been induced by the acid formed. Gorini³ found

¹ Archiv für Hygiene, Bd. xxxi, p. 311.

² Ibid., Bd. xi, and Centralblatt für Bacteriologie, Bd. xii.

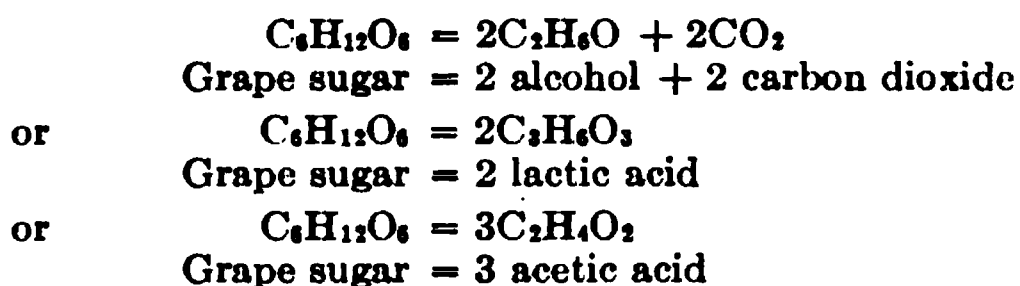
³ Centralblatt für Bacteriologie, Bd. xii, p. 666.

that cultures of *bacillus prodigiosus*, sterilized by heating to 60° C., caused a solid coagulation of sterile milk in a few days.

A small number of bacteria have also been encountered that bring about coagulation of milk with a distinctly alkaline reaction. This function has been noticed in bacteria isolated from milk, and especially in *bacterium pseudodiphtheriticum* isolated from cows' milk (Bergey).

Sugar-splitting enzymes are very common in bacteria. This function varies in different species as seen in the different end-products that are formed. Buchner succeeded in isolating the sugar-splitting enzyme (zymase) of yeast-cells, and when thus isolated it still possesses the power of inducing active fermentation of sugar. It is believed that the sugar-splitting enzymes of bacteria are similar in character to the zymase of yeast-cells. The splitting up of carbohydrates appears to be brought about by the bacteria for the purpose of obtaining oxygen, as indicated by the nature of the end-products formed, and also by the conditions under which it may be carried out—*i. e.*, the absence of atmospheric oxygen.

The splitting of the carbohydrate molecule may be illustrated as follows:



According to Theobald Smith¹ all facultative anaërobic bacteria² form acids from carbohydrates, while the strictly

¹ Centralblatt für Bacteriologie, Bd., xviii.

² See "aërobic" and "anaërobic" bacteria.

aërobic bacteria do not have this function, or bring about the alteration so slowly that it is concealed by the simultaneous production of alkali. Among the acids formed by bacteria, besides carbon dioxide, we have lactic, acetic, butyric, propionic, and formic; and frequently there is also produced ethyl alcohol, aldehyde, and acetone.

The lactic acid formed by the action of different bacteria on carbohydrates may be either dextrorotatory or levorotatory, or almost equal quantities of both forms may be present and the mixture be optically inactive.

Bacterial Proteins.—The proteid matter making up the bodies of many species of bacteria, even those not conspicuously pathogenic, was shown by H. Buchner to induce disease when isolated and injected into the tissues of animals; in some cases causing only slight fever, in others acute inflammation with suppuration. For such compounds he suggested the name "bacterial proteins."

Ptomains.—Ptomains, or as they are sometimes called "putrefactive alkaloids" or "cadaveric alkaloids," are crystallizable, nitrogenous bodies that are the results of bacterial action upon dead organic matter. They differ from enzymes in that they are the occasional results of defective bacterial metabolism and from both toxins and enzymes in that they are crystallizable and of definite chemical composition. Some of them are poisonous, many are not. The conditions favorable to the elaboration of ptomains by bacteria vary, but in the main the most poisonous of the ptomains appear to be the result of bacterial activity under a limited supply of oxygen. Poisonous ptomains are sometimes formed within the intestinal canal of man either as a result of malfermentation or of interruption of normal oxidation. We have no reason for believing that

ptomains play any part in either the causation or course of the definite, infective diseases.

Ptomains have been isolated from decomposing cadavers, from putrid meat, milk, cheese, and from a number of bacterial cultures. Poisonous ptomains occasionally develop in improperly preserved food. True toxins and dangerous bacteria have also been found in such substances.

Nutrition of Bacteria.—We have said that through the agency of chlorophyl, in the presence of sunlight, the green plants are enabled to obtain the amount of nitrogen and carbon which is necessary to their growth from such simple bodies as carbon dioxide and ammonia, which they decompose into their elementary constituents. The bacteria, on the other hand, owing to the absence of chlorophyl from their tissues, do not possess this power. They must, therefore, have their carbon and nitrogen presented as such, in the form of decomposable organic substances.

In general, bacteria obtain their nitrogen most readily from soluble proteins, and to a certain extent, but by no means so easily, from salts of ammonium. In some of Nägeli's experiments it appeared probable that they could obtain the necessary amount of nitrogen from inorganic nitrates. At all events, he was able in certain cases to demonstrate a reduction of nitric to nitrous acid and ultimately to ammonia. Nevertheless, in all of these experiments circumstances point to the probability that the nitrogen obtained by the bacteria for building up their tissues in the course of their development was derived from some source other than the nitric acid or the nitrates, and that the reduction of this acid was most probably a secondary phenomenon. We must bear in mind, however, the specific group, the nitrifying bacteria, which increase and mul-

tiply without appropriating proteid nutrition. They are, as stated above, concerned in the particular form of fermentation that results in the oxidation of ammonia to nitrous and nitric acids, a process everywhere in progress in the superficial layers of the soil.

For the supply of carbon many of the carbon compounds serve as sources upon which the bacteria can draw. The carbon deficit, for example, can be obtained from sugar and bodies of like composition; from glycerin and many of the fatty acids; and from the alkaline salts of tartaric, citric, malic, lactic, and acetic acids. In some instances carbon compounds, which when present in concentrated form inhibit the growth of bacteria, may, when highly diluted, serve as nutrition for them. Salicylic acid and ethyl alcohol are of this class.

In addition to carbon and nitrogen, water is essential to the life and development of bacteria; without it no development occurs, and in many cases drying kills them. Certain species and developmental forms, on the contrary, though incapable of multiplying when in the dry state, may be dried without causing them to lose the power of reproduction when again placed under favorable conditions.

Closer study of bacteria, and a more intimate acquaintance with their nutritive changes, demonstrate an appreciable variability in the character of the substances best suited for the nutrition of different species, as well as in the end products of such nutrition, for instance: one species may require a tolerably concentrated form of nutrition, while another needs but a very limited amount of proteid substance for its development; some bring about profound alterations in the media in which they are growing, while others produce but little apparent change; for certain species

free oxygen is essential, for others it is harmful. In one case alterations in the reaction of the media will be conspicuous, while in another no such variation can be detected. As shown above the growth of some species is accompanied by evidence of specific fermentations; of others by the appearance of poisonous; of others by putrefactive changes.

In considering the normal development of bacteria we must not lose sight of the fact that this is influenced both by the quality and the quantity of the nutritive materials to which they have access, and by the character of the metabolic products that accumulate in these materials as a result of their vital processes. Nitrogen and carbon compounds may be present in amount and kind entirely suitable to normal bacterial growth, and yet this may be checked, after a comparatively short time, by the accumulated products of bacterial metabolism, some of which possess the property of inhibiting growth and ultimately of even destroying the bacteria that produced them. The most common and conspicuous examples of such inhibiting conditions is alteration in the chemical reaction of the media in which the bacteria are developing.

In the case of a number of species there begins, coincidently with retardation of normal development, a process of dissolution, self-digestion or "autolysis," which may continue until the cells are unrecognizable as bacteria. This phenomenon is the result of the action of enzymes located within the cells which, under normal conditions of growth, are concerned in the life processes of the cell, but which, on the advent of conditions unfavorable to the growth and multiplication of the cells, react upon them and cause their actual solution. An analogous "autolysis" is often to be seen with animal cells. If bits of living tissue be removed

from the body, under aseptic precaution, and kept at suitable conditions of moisture and temperature they may ultimately become completely liquefied as a result of the digestive action of hydrolysing enzymes contained within them.

Their Relation to Oxygen.—Of primary importance and interest in the study of the nutritive changes of bacteria is the difference in their relation to oxygen. For certain species free oxygen is essential to the proper performance of their functions; in another group no evidence of life can be detected under its access; while in a third group free oxygen appears to play but an unimportant role, for development occurs as well with as without it. It was Pasteur who first demonstrated the existence of particular species of bacteria which not only grow and multiply and perform definite physiological functions without the aid of free oxygen, but to the existence of which it is positively harmful. To these he gave the name *anaërobic* bacteria, in contradistinction to the *aërobic* group, for the proper performance of whose functions free oxygen is essential. In addition to these there is a third group, for the maintenance of whose existence the absence or presence of uncombined oxygen is apparently of no moment—development progresses as well with as without it; the members of this group comprise the class known as *facultative* in their relation to this gas. It is to this third group, the facultative, that the majority of bacteria belong. Since all growing bacteria, anaërobic as well as aërobic, generate carbonic acid in the course of their development, it is evident that oxygen must in reality be obtained by them from some source, and must be regarded as essential to their life-processes; but the manner in which it is appropriated by them varies, the aërobic species taking

it from the air as free oxygen, while the anaërobic species, not possessed of this ability, obtain it through the decomposition of more or less stable oxygen-containing compounds.

Though the multiplication of the facultative varieties is not interfered with by either the presence or absence of free oxygen, yet experiments demonstrate that the products of their growth are different under the varying conditions of absence or presence of this gas. For example: in the case of certain of the chromogenic forms the presence or absence of oxygen has a very decided effect upon the production of the pigments by which they are characterized.

NOTE.—Observe the difference between the intensity of color produced upon the surface of the medium and that along the track of the needle in stab-cultures of *bacillus prodigiosus* and of *spirillum rubrum*. In the former the red color is apparently a product dependent upon the presence of oxygen, while in the latter the greatest intensity of color occurs at the point furthest removed from the action of oxygen.

Influence of Temperature upon the Growth.—Another factor which plays a highly important part in the biological functions of these organisms is the temperature under which they exist. The extremes of temperature between which the majority of bacteria are known to grow range from 5.5° to 43° C. At the former temperature development is hardly appreciable; it becomes more and more active until 38° C. is reached, when it is at its optimum, and, as a rule, ceases at 43° C.; though species exist that multiply at as high a temperature as 70° C. and others at as low as 0° C. The

investigations of Globig,¹ Miquel,² and Macfayden and Bloxall³ have revealed the existence in the soil, in water, in feces, in sewage, in dust, and, in fact, practically everywhere, of bacteria that under artificial cultivation show no evidence of life at a temperature lower than 60° to 65° C., and will even grow at such high temperatures as 70° and 75° C., a state of affairs almost paradoxical, inasmuch as these are temperatures that suffice for the coagulation of albumin, and, in consequence, are generally incompatible with life. Rabinowitsch⁴ has likewise described a number of species of these *thermophilic* bacteria, as they are called; but states that it was possible in her experiments to obtain evidence of their growth at the lower temperature (34° to 44° C.), as well as at the higher temperature mentioned by preceding investigators. It is possible that this peculiarity is but a manifestation of adaptation to environment and not an essential to the life processes of these species.

The most favorable temperature for the development of pathogenic bacteria is that of the human body, viz., 37.5° C. There are a number of bacteria commonly present in water, the so-called normal *water bacteria*, that grow best at about 20° C.

Coöperating Bacteria.—Under natural conditions it frequently occurs that the development of one species or group of species of bacteria is directly dependent upon the functional activities of another totally distinct species, the growth of one group resulting in conditions that are of vital importance to the existence of the other. Such interdepen-

¹ Zeitschrift für Hygiene, Bd. iii, S. 294.

² Annales de Micrographie, 1888, pp. 4 to 10.

³ Journal of Path. and Bact., vol. iii, Part I.

⁴ Zeitschrift für Hygiene u. Infektionskrankheiten, Bd. xx, Heft. 1, S. 154 to 164.

dence is observed, for instance, in complete nitrification, as already noted; in the course of putrefaction, where, through exhaustion of free oxygen by the actively germinating aërobic varieties, the conditions are supplied that enable the anaërobic species to develop and exercise their biological activities. Again, through the proteolytic activity of enzymes produced by certain species of bacteria, other species are supplied with nutrition that would otherwise be unassimilable or only imperfectly so. Similar coöperative or symbiotic relations between bacteria and higher plants are also noticed, notably that between certain bacteria of the soil and the group of leguminous plants, whereby the latter are enabled, through the assistance of the former, to make up their nitrogen deficit in large part from the free nitrogen of the atmosphere. This latter relationship is probably an example of true *symbiosis*.¹

Influence of Light.—Light is not only unnecessary to the performance of functions by bacteria but appears to be in varying degrees inhibitory.

Direct sunlight is destructive to many species. It is a matter of common experience that cultures of particularly important species retain their type characteristics better and longer if cultivated in the dark than in diffuse daylight.

Electric light has likewise a depressing influence upon the viability of bacteria. Beyond the fact that bacteria *in vacuo* are unaffected by light we have no knowledge of the mechanism of its action. Presumably it has something to do with oxidation processes.

The germicidal action of the direct rays of the sun may be easily demonstrated by preparing a plate of colon bacillus, shading a portion and allowing the sun to shine upon it for

¹ See Nitrogen fixing bacteria.

a time, varying with the intensity of its light. Growth will occur in the shaded part, none or only relatively little in the illuminated part of the plate.

Influence of Pressure.—The influence of pneumatic pressure on the viability of bacteria appears to depend upon the character of the gas used. Ordinary air, or its constituents, oxygen and nitrogen, whenever pressed heavily (600 to 2000 atmospheres) upon cultures of bacteria, have a slight inhibitory effect. Carbon dioxide under five to ten atmospheres pressure is shown by Park and his associates to destroy almost all of the typhoid, dysentery, diphtheria and colon bacilli exposed to it within twenty-four hours.

Effect of Moisture.—As is the case with all living plants a degree of moisture is essential to life. Certain species of bacteria are killed by ordinary drying, and many of them by absolute drying. The spores (to be described later) of bacteria are not so effected, a few species retaining their power to germinate after having been dried, as the word is ordinarily understood, for a comparatively long time, and spores have been kept in a dry state for years without losing their power to germinate.

Influence of Electricity.—The methods employed for deciding this point have led to results that are inconclusive and not easy of interpretation.

It is true that when bacteria are exposed to the electric current they are often inhibited and sometimes killed.

This result may be interpreted in several ways, viz.: The elevation of temperature caused by the current may explain the destruction; the electrolytic action of the current on matters in which the bacteria are located may, by dissociation, liberate agents that are destructive to bacteria, or a

similar destructive dissociation within the bacteria themselves may result from the action of the current.

The evidence at hand does not permit of the acceptance of either of these suggestions as the correct interpretation of the results.

Chemotaxis.—Another interesting biological peculiarity of bacteria is that discovered by Engelmann and by Pfeffer, known as *chemotaxis*. This term applies to the peculiar phenomena of attraction and of repulsion that are exhibited by motile bacteria when in the presence of solutions of bodies of various chemical composition. It was demonstrated that the bacteria in decomposing infusions accumulate in great numbers in the neighborhood of the sources of oxygen. In a hanging-drop of such an infusion the bacteria will be seen to accumulate in a dense mass along the margin or around the edge of small bubbles of air in the fluid. Even plant cells in the infusion, whose chlorophyl sets free oxygen in the light, are surrounded by large numbers of bacteria. The positive chemotactic affinity between oxygen and bacteria was employed by Engelmann as a basis for the demonstration of small quantities of oxygen in studying the influence of various kinds of light upon the assimilation of green plant-cell. Pfeffer showed that when a neutral fluid (a drop of water) containing motile bacteria is brought in contact with a weak solution of either peptone, sodium chloride, or dextrin, the bacteria are at once attracted toward the solution; this reaction is designated "positive chemotaxis." On the other hand, if brought in contact with an acid, an alkaline, or an alcoholic solution, the bacteria are repelled or driven from the point at which the two fluids are diffusing; that is, they exhibit "negative chemotactic" affinities. The significance of these reactions is not under-

stood, but it has been aptly suggested that they may be fundamentally analogous to the specific positive and negative affinities exhibited by the ions resulting from dissociation of electrolytes, and that they may "have their explanation in the forces of ionic attraction and repulsion." In this connection it is important to note that the wandering cells of the animal body, the leucocytes, exhibit also these chemotactic phenomena; and it is especially necessary to a complete comprehension of the process of suppuration to bear in mind that among the substances which have the greatest attraction for these wandering cells, are the products of growth of certain bacteria in some cases, and the protein constituents of the bacteria themselves in others.

To summarize briefly the foregoing it may be said, in general, that for the growth and development of bacteria nitrogenous organic matter of a neutral or slightly alkaline reaction, in the presence of moisture and at a suitable temperature, is all that is necessary. From this can be formed some idea of the omnipresence in nature of these minute vegetables. Bacteria are found wherever these conditions obtain.

¹ Read Sewall on Some Relations of Osmosis and Ionic Action in Clinical Medicine, International Clinics, vol. xi, Eleventh Series.

CHAPTER II.

Morphology¹ of Bacteria—Chemical Composition of Bacteria—Mode of Multiplication—Spore-formation—Motility.

IN structure the bacteria are unicellular, always developing from pre-existing cells of the same character and never appearing spontaneously. They are seen to occur as spherical, rod- and spiral-shaped bodies that multiply by the simple process of transverse division, belonging, therefore, to the *schizomycetes* or fission fungi.

In size the bacteria are among the smallest living creatures with which we are acquainted, being visible only when very highly magnified. In order that some conception of their microscopic dimensions may be formed, it has been computed that of the average size bacteria about thirty billion would be required to weigh a gram, and that about one billion seven hundred million of the small spherical forms might readily be suspended in a drop of water.

Under what we are accustomed to regard as normal conditions of development, and by the ordinary methods of examination, bacteria appear very simple in form and structure. They are cells consisting of a protoplasmic mass within a membranous hull that is discernible with more or less difficulty. The protoplasmic body is of material closely allied, chemically speaking, to ordinary vegetable protein. It is often homogenous, but in particular species and under various conditions of growth the central mass is stained

¹ Morphology, pertaining to shape, outline, structure.

specimens is commonly marked by the presence of very dark granules, the so-called metachromatic granulations. Again, in other species paraplastic granules giving the microchemical reactions of fat, starch, sulphur, etc., are to be seen. Under certain physical conditions the protoplasmic body presents irregular rents or retractions, the result of proteolytic or of osmotic disturbances dependent upon the character of the fluid in which the bacteria are located; in fact, the deeply staining granules, other than those of fat, starch, and sulphur, that are often observed, are regarded by some writers (especially A. Fischer) as but altered or condensed protoplasm due to the same influences.

In certain species the protoplasmic body is always more dense at the poles of the cells than at the middle, so that when stained the ends are much darker than the intervening portion. In other species the reverse is the case.

By some investigators the protoplasmic central mass is regarded as a nucleus, and, functionally speaking, possibly it is to all intents and purposes, but this cannot be certainly decided. In the great majority of cases, however, with the ordinary methods of examination, it is not seen to possess any of the structural peculiarities that we are accustomed to regard as the distinguishing attributes of cell-nuclei.

The enveloping hull or membrane is in some cases apparently only a modification of the protoplasmic central mass, at times being only a condensation of that protoplasm; again, it seems to be chemically different from it. In a few instances it appears to be allied to cellulose in its chemical composition. Sometimes it is so thick as to be readily seen, while again it is discernible only by special methods of examination. In particular species it may, by appropriate methods, be seen as a sharply defined capsule inclosing a

clear zone in which the deeply stained central mass lies. Occasionally the central protoplasmic mass is surrounded by an ill-defined slimy material that causes the individual cells to adhere to one another in more or less compact masses or pellicles (zoöglea, Fig. 1).

Chemical Composition of Bacteria.—The bodies of bacteria consist of water, salts, and albuminous substances, with smaller proportions of various extractives soluble in alcohol or ether, such as triolein, tripalmitin, tristearin, lecithin, and cholesterin. In many varieties substances giving the reaction of starch have been found, while others give the true reactions of cellulose (*B. subtilis*). Nuclein has not

FIG. 1

Zoöglea of bacilli.

been found in any of the bacteria, though the nuclein bases, xanthin, guanin, adenin, have been found.

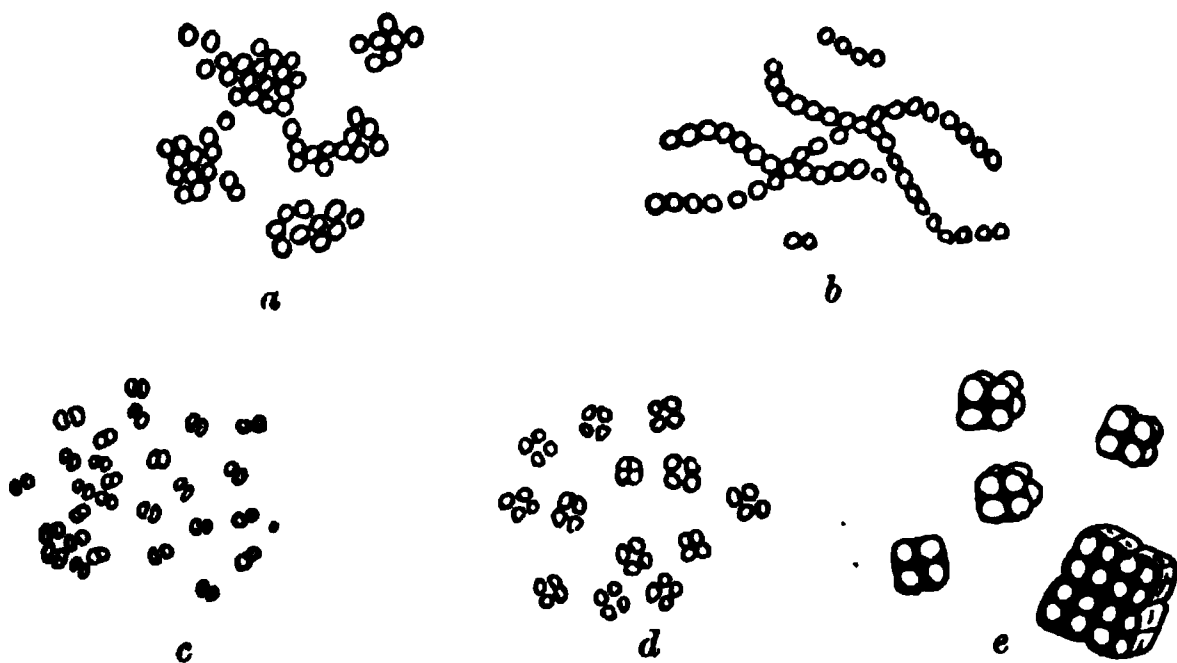
The relative amounts of water in bacteria are influenced to a large extent by the nature of the medium on which they have been grown. In like manner the content in albumin, extractive substances, and salts varies with the conditions under which the bacteria have been cultivated. E. Cramer¹ has studied the chemical composition of bacteria in great detail. As the result of his studies of *microspira comma*, he found its composition to be as follows: water

¹ Archiv für Hygiene, Bd. xiii, xvi, xxii, and xxviii.

88.3 per cent., albumin 7.6 per cent., ash 3.6 per cent. The dry substance of the bacteria contains the following: albumin 65 per cent., ash 31 per cent. From 76 to 80 per cent. of the ash consists of sodium chloride and phosphate.

Morphology of Bacteria.—For the purposes of this book it will suffice to classify the bacteria roughly into three morphological groups with their subdivisions, the members of each group being identified by their individual outline, viz., that of a sphere, a rod, or a spiral. To these three grand

FIG. 2



a, staphylococci; *b*, streptococci; *c*, diplococci; *d*, tetrads; *e*, sarcinae.

divisions are given the names cocci or micrococci, bacilli, and spirilla.

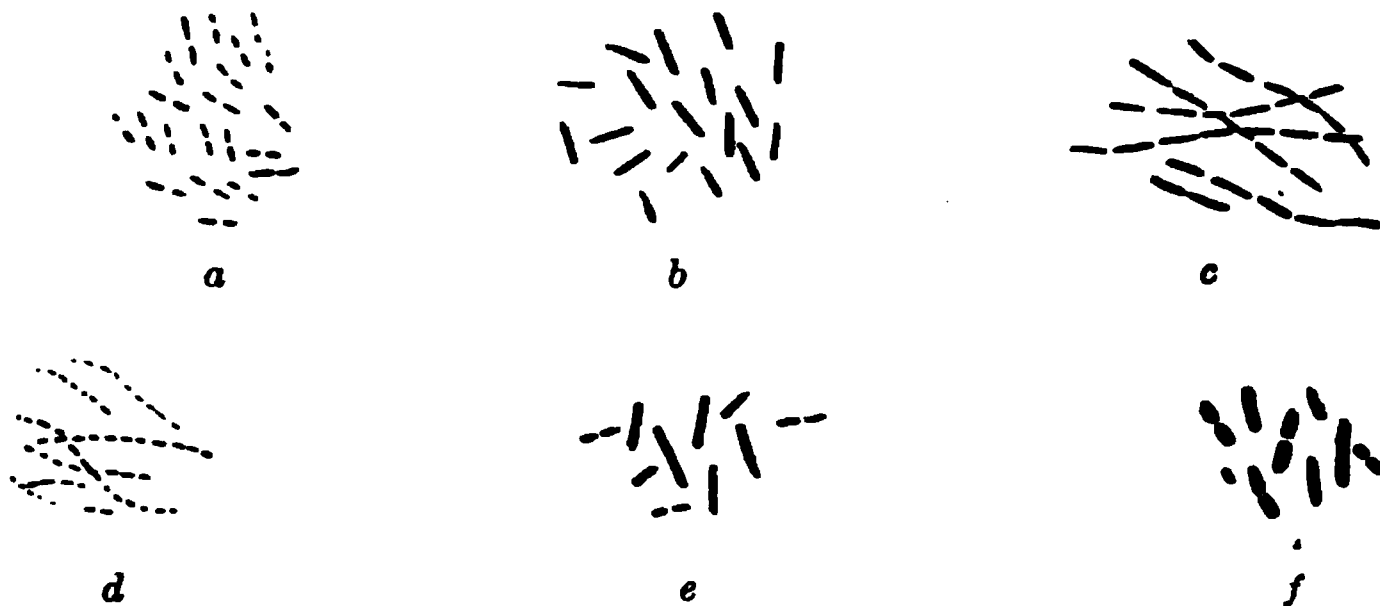
Mode of Multiplication.—In the group *micrococci* belong all spherical forms—*i. e.*, all those forms the isolated individual members of which are practically of the same diameter in all directions. (See Fig. 2, *a*, *b*, *c*, *d*, *e*.)

The *bacilli* comprise all oval or rod-formed bacteria. (See Fig. 3.)

To the *spirilla* belong the bacteria that are curved when

seen in short segments and that appear as undulating threads when such segments are of greater length or when several short segments are joined end to end. (See Fig. 4.)

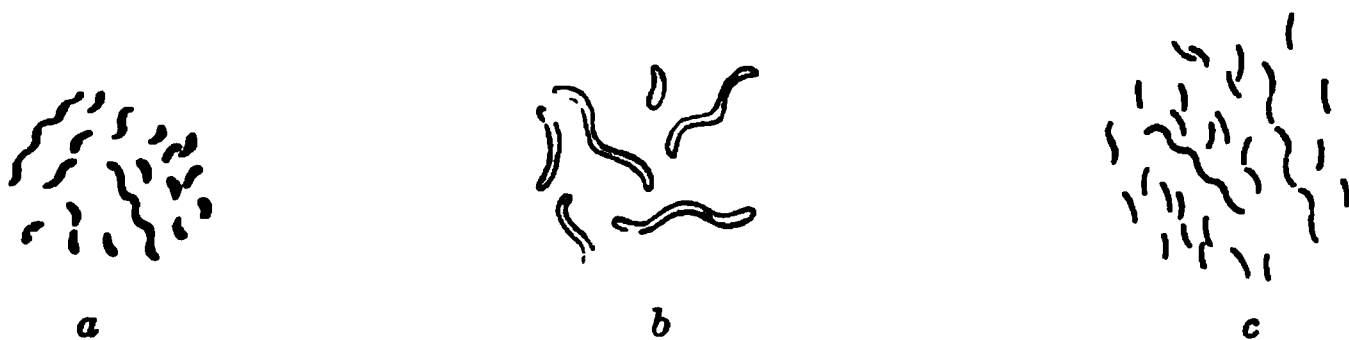
FIG. 3



a, bacilli in pairs; *b*, single bacilli; *c* and *d*, bacilli in threads; *e* and *f*, bacilli of variable morphology.

The micrococci are subdivided according to their prevailing mode of grouping, as seen in growing cultures, into *staphylococci*—those growing in masses like clusters of grapes

FIG. 4



a and *c*, spirilla in short segments and longer threads—the so-called comma forms and spirals; *b*, the thick spirals sometimes known as vibrios.

(see Fig. 2, *a*); *streptococci*, those growing in chains consisting of a number of individuals strung together like beads upon a string (see Fig. 2, *b*); *diplococci*—those growing

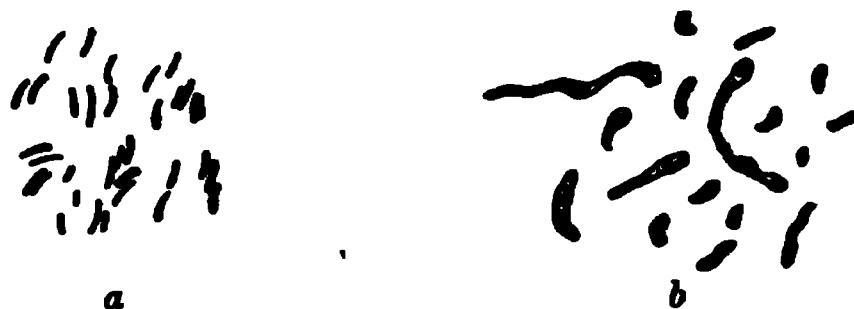
in pairs (Fig. 2, *c*); *tetrads*—those developing as fours (Fig. 2, *d*); and *sarcinæ*—those dividing into fours, eights, etc., as cubes—that is, in contradistinction to all other forms, the segmentation, which is rarely complete, takes place regularly in three directions of space, so that when growing the bundle of segmenting cells presents somewhat the appearance of a bale of cotton (Fig. 2, *e*).

To the bacilli belong all straight, oval and rod-shaped bacteria—*i. e.*, those in which one diameter is always greater than the other. In this group are found those organisms the life-cycle of many of which presents deviations from the simple rod shape. Many of them in the course of development increase in length into long threads, along which traces of segmentation may usually be found. Again, under certain conditions, many of them possess the property of forming within the body of the rods oval, glistening spores (see Fig. 6), and, if the conditions are not altered, the rods may entirely disappear and nothing be left in the culture but these oval spores. In some of them this phenomenon of spore-formation is accompanied by an enlargement or swelling of the bacillus at the point at which the spore is located (see Fig. 6, *c* and *d*). Again, many of them, from unfavorable conditions of nutrition, aëration, or temperature, undergo pathological changes that are probably autolytic in nature—that is, the individuals themselves experience degeneration of their protoplasm with coincident distortion of their outline; they are then usually referred to as “involution-forms” (see Fig. 5, *a* and *b*). In all of these conditions, however, so long as death has not occurred, it is possible to cause these forms to revert to the typical rods from which they originated, by the renewal of conditions favorable to their normal vegetation.

It must be borne in mind, though, that it is never possible by any means to bring about changes in these organisms that will result in the permanent conversion of the morphology of the members of one group into that of another—that is, one can never produce bacilli from micrococci, nor *vice versa*; and any evidence which may be presented to the contrary is based upon untrustworthy methods of experimentation.

Very short oval bacilli may sometimes be mistaken for micrococci, and at times micrococci in the stage of segmentation into diplococci may be mistaken for short bacilli; but

FIG. 5



a, spirillum of Asiatic cholera (comma bacillus); normal appearance in fresh cultures; *b*, involution-forms of this organism as seen in old cultures.

by careful inspection it will always be possible to detect a continuous outline along the sides of the former, and a slight transverse indentation or partition-formation between the segments of the latter. The high index of refraction of spores, the property which gives to them their glistening appearance, will always serve to distinguish them from micrococci. This difference in refraction is especially noticeable if the illumination of the microscope be reduced to the smallest possible bundle of light-rays. The spores, moreover, take up staining-reagents much less readily than do the micrococci. The most reliable differential points,

however, are the infallible properties possessed by the spores of developing into bacilli, and by the spherical organism with which they may have been confounded of always producing other micrococci of the same spherical form.

We have less knowledge of the life-history of the spiral forms. Efforts toward their cultivation under artificial conditions have thus far been successful in only a comparatively limited number of cases. Morphologically, they are thread- or rod-like bodies which are twisted into the form of spirals. In some of them the turns of the spiral are long, in others quite short. In some the threads appear rigid, in others flexible. They are motile and multiply apparently by the simple process of fission.¹

Mode of Multiplication.—The micrococci multiply by simple fission. When development is in progress a single cell will be seen to elongate slightly in one of its diameters. Over the centre of the long axis thus formed will appear a slight indentation in the outer envelope of the cell; this indentation will increase in extent until there exist eventually two individuals which are distinctly spherical, as was the parent from which they sprang, or they will remain together for a time as diplococci; the surfaces now in juxtaposition are flattened against one another, and not infrequently a fine, pale dividing-line may be seen between the two cells. (See Fig. 2, *c* and *d*.) A similar division in the other direction will now result in the formation of fours as tetrads.

In the formation of staphylococci such division occurs irregularly in all directions, resulting in the production of

¹ Dividing into two transversely.

the clusters in which these organisms are commonly seen. (See Fig. 2, *a*.) With the streptococci, however, the tendency is for the segmentation to continue in one direction only, resulting in the production of long chains of 4, 8, and 12 individuals. (See Fig. 2, *b*.)

The sarcinæ divide more or less regularly in three directions of space; but instead of becoming separated the one from the other as single cells, the tendency is for the segmentation to be incomplete, the cells remaining together in masses. The indentations upon these masses or cubes, which indicate the point of incomplete fission, give to the bundles of cells the appearance commonly ascribed to them, viz., that of a bale of cotton or a packet of rags. (See Fig. 2, *e*.)

The mode of multiplication of bacilli is similar to that of the micrococci—*i. e.*, a dividing cell elongates slightly in the direction of its long axis; an indentation appears about midway between its poles, and this becomes deeper and deeper, until eventually two daughter-cells have formed. This process may occur in such a way that the two young bacilli adhere together by their adjacent ends in much the same way that sausages are seen to be held together in strings (Fig. 3, *f*), or the segmentation may take place more at right angles to the long axis, so that the proximal ends of the young cells are flattened, while the distal extremities may be rounded or slightly pointed (Fig. 3, *e*). The segmentation of the anthrax bacillus, with which we are to become acquainted later, results, when completed, in an indentation of the adjacent extremities of the young segments, so that by the aid of high magnifying powers these surfaces are seen to be actually concave. Bacilli never divide longitudinally.

Spore-formation.—With the spore-forming bacilli, under favorable conditions of nutrition and temperature, the same mode of segmentation is seen to occur during vegetation; but as soon as these conditions become altered by the exhaustion of nourishment, the presence of detrimental substances, unfavorable temperatures, etc., they enter, in their life-cycle, the stage to which we have referred as *spore-formation*. This is the process by which the organisms are enabled to enter a state in which they resist deleterious influences to a much higher degree than is possible for them when in the growing or vegetative condition.

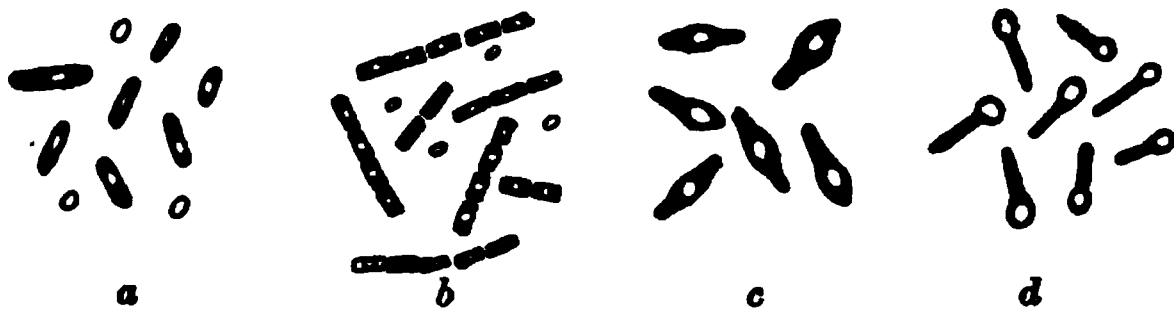
In the spore, dormant, or permanent state, as it is variously called, no evidence of life whatever is given by the spores; though as soon as the conditions which favor their germination have been renewed these spores develop again into the same kind of cells as those from which they originated, and the appearances observed in the vegetative or growing stage of their history are repeated.

Multiplication of spores, *as such*, does not occur; they possess only the power of developing into individual rods of the same nature as those from which they were formed, *but not of giving rise to a direct reproduction of spores*.

When the conditions which favor spore-formation present, the protoplasm of the vegetative cells is seen to undergo a change. It loses its normal homogeneous appearance and becomes marked by granular, refractive points of irregular shape and size. These eventually coalesce, leaving the remainder of the cell clear and transparent. When this coalescence of highly refractive particles is complete the spore is perfected. In appearance the spore is oval or round, and very highly refractive—glistening. It is easily differentiated from the remainder of the cell, which now consists

only of a cell-membrane and a transparent, clear space which surrounds the spore. Eventually both the cell-membrane and its fluid contents disappear, leaving the oval spore free; it then gives the impression of being surrounded by a dark, sharply defined border. When thus perfectly developed, the spore may be regarded as analogous to the seeds of higher plants. Like the seed, it evinces no evidence of life until placed under conditions favorable to germination, when there develops from it a cell identical in all respects with that from which it originated. Its tenacity of life, as in the case of seeds, is almost unlimited. It may

FIG. 6



a, *Bacillus subtilis* with spores; *b*, *Bacillus anthracis* with spores; *c*, *Clostridium* form with spores; *d*, *Bacillus* of tetanus with end spores.

be kept in a dry state, and this has actually been done, for years without loss of viability.

The glistening, enveloping spore-membrane is not of uniform thickness throughout, and in consequence when germination occurs the growing bacillus, the so-called vegetative form of the organism, protrudes through the thinnest part of the spore-membrane—that is, through the point of least resistance. This may be either the end or the side of the spore, according to the species under observation. In certain cases such a protrusion is not observed, but in its place the spore *in toto* appears to be gradually absorbed or

in some way converted directly into a vegetating cell. It evinces no motion other than the mechanical tremor common to all insoluble microscopic particles suspended in fluids, and it remains quiescent until there appear conditions favorable to its subsequent development.

By the ordinary methods of staining, spores do not become colored, so that they appear in the stained cells as pale, transparent, oval bodies, surrounded by the remainder of the cell, which has taken up the dye.

A single cell produces but one spore. This may be located either at an extremity or in the centre of the cell (Fig. 6).

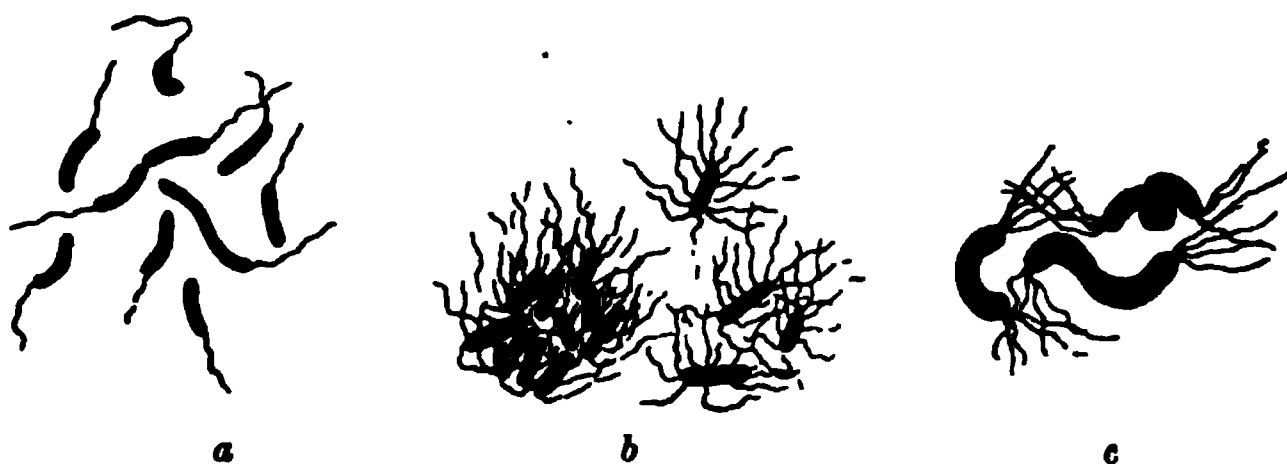
Occasionally spore-formation is accompanied by an enlargement of the cell at the point at which the process is in progress. As a result, the cell loses its regular rod shape and becomes that of a club, a drum-stick, or a lozenge, depending upon whether the location of the spore is to be at the pole or in the centre of the cell. (See Fig. 6, *c* and *d*.)

Motility.—In addition to the property of spore-formation there is another striking difference between various species of bacteria, namely, the property of *motility*, by which some of them are distinguished. This power of motion is due to very delicate, hair-like appendages or flagella, by the lashing motions of which the cells possessing them are propelled through the fluid. In some cases the flagella are located at but one end of the organism, either singly (monotrichic) or in a tuft (lophotrichic); and in some cases, especially of the bacillus of typhoid fever, they are given off from the whole surface of the rod (peritrichic). (See Fig. 7.)

For a long time this property of independent motion could only be assumed to be due to the possession of some such form of locomotive apparatus, because similar appendages

had been seen upon some of the large motile spirilla found in stagnant water, but it was not until a few years ago that the accuracy of this assumption was actually demonstrated. By a special method of staining Löffler¹ rendered visible these hair-like appendages. His method, as well the several

FIG. 7



a, spiral forms with a flagellum at only one end; b, bacillus of typhoid fever with flagella given off from all sides; c, large spirals from stagnant water with wisps of flagella at their ends (*spirillum undula*).

modifications that have been made of it, depends for success upon the use of mordants, through the agency of which the stains employed are caused to adhere with increased tenacity to the objects under treatment.

¹ Löffler's method of staining will be found in the chapter devoted to this part of the technique.

CHAPTER III.

Principles of Sterilization by Heat—Methods Employed—Discontinued Sterilization—Fractional Sterilization—Apparatus Employed—Sterilization under Pressure—Sterilization by Hot Air—Thermal Death-point of Bacteria—Chemical Disinfection and Sterilization—Mode of Action of Disinfectants—Practical Disinfection.

OF fundamental importance to successful bacteriological manipulations are acquaintance with the principles underlying the methods of sterilization and disinfection, and familiarity with the approved methods of applying these principles in practice.

In many laboratories it is customary to employ the term sterilization for the destruction of bacteria by heat, and the term disinfection for the accomplishment of the same end through the use of chemical agents. Such distinction in the use of the terms is obviously incorrect, as we shall endeavor to explain.

The laboratory application of the word sterilization for the destruction of bacteria by high temperatures probably arose from the circumstance that culture-media, and certain other articles that it is desirable to render free from bacterial life, are not treated by chemical agents for this purpose, but are exposed to the influence of heat in various forms of apparatus known as sterilizers; and the process is, therefore, known as sterilization. On the other hand, cultures no longer useful, bits of infected tissue, and apparatus generally that it is desirable to render free from danger, are commonly subjected for a time to the action of chemical compounds possessing germicidal properties—*i. e.*, to the action

of disinfectants; and the process is, consequently, known as disinfection, though the same end can also be reached by the application of heat to these articles. Strictly speaking, sterilization implies the *complete* destruction of the vitality of all micro-organisms that may be present in or upon the substance to be sterilized, and can be accomplished by the proper application of both thermal and chemical agents; while disinfection, though it may insure the destruction of *all* living forms that are present, need not of necessity do so, but may be limited in its action to those only that possess the power of *infecting*; it may or may not, therefore, be complete in the sense of sterilization. From this we see it is possible to accomplish both sterilization and disinfection as well by chemical as by thermal means.

In practice the employment of these means is governed by circumstances. In the laboratory it is essential that all culture-media with which work is to be conducted should be free from living bacteria or their spores—they must be sterile; and it is equally important that their original chemical composition should remain unchanged. It is evident, therefore, that sterilization of these substances by means of chemicals is out of the question, for, while the media *could* be thus sterilized, it would be necessary, in order to accomplish this, to add to them substances capable not only of destroying all micro-organisms present, but whose presence would at the same time prevent the growth of bacteria that are to be subsequently cultivated in these media—that is to say, after performing their sterilizing or germicidal function the chemical disinfectants would, by their further presence, exhibit their *antiseptic* properties and thus render the material useless as a culture-medium. Exceptions to this are seen, however, in the case of certain

volatile substances possessing disinfectant powers—chloroform and ether, for instance; these bodies, after exhibiting their germicidal activities, may be driven off by gentle heat, leaving the media quite suitable for purposes of cultivation. They are not, however, in general use in this capacity.

The circumstances under which *chemical* sterilization or disinfection is practised in the laboratory are, ordinarily, either those in which it is desirable to render materials free from danger that are not affected by the chemical action of the agents used, such as glass apparatus, etc., or where destructive changes in the composition of the substances to be treated, as in the case of old cultures, infected tissues, pathological exudates, feces, etc., are a matter of no consequence. On the other hand, for the sterilization of all materials to be used as culture-media heat only is employed.¹

The two processes will be explained in this chapter, beginning with

STERILIZATION BY HEAT.

Sterilization by means of high temperature is accomplished in several ways, viz., by subjecting the articles to be treated to a high temperature in a properly constructed oven—this is known as *dry* sterilization; by subjecting them to the action of streaming or live steam at the temperature of 100° C.; and by subjecting them to the action of steam under pressure, under which circumstance the temperature to which they are exposed becomes more and more elevated as the pressure increases.

Experience has taught us that the process of sterilization by dry heat is of limited application because of its many

¹ An occasional exception to this is the use of chloroform, mentioned above.

disadvantages. For successful sterilization by the method of dry heat, not only is a relatively high temperature needed, but the substances under treatment must be exposed to this temperature for a comparatively long time. The penetration of dry heat into materials which are to be sterilized is, moreover, much less thorough than that of steam. Many substances of vegetable and animal origin are rendered valueless by subjection to the dry method of sterilization. For these reasons comparatively few materials can be sterilized in this way without seriously impairing their further usefulness.

Successful sterilization by dry heat cannot usually be accomplished at a temperature lower than 150° C., and to this degree of heat the objects should be subjected for not less than one hour. For the sterilization, therefore, of the organic materials of which the media employed in bacteriological work are composed, and of domestic articles, such as cotton, woollen, wooden, and leather articles, this method is wholly unsuitable. In bacteriological work its application is limited to the sterilization of glassware principally—such, for example, as flasks, plates, small dishes, test-tubes, pipettes—and such metal instruments as are not seriously injured by the high temperature.

Methods Employed.—Sterilization by moist heat—steam—offers conditions much more favorable. The penetrating power of the steam is not only more energetic, but the temperature at which sterilization is ordinarily accomplished is, as a rule, not destructive to the objects under treatment. This is conspicuously seen in the work of the laboratory; the culture-media, composed in the main of decomposable organic materials that would be rendered entirely worthless if exposed to the dry method of sterilization, sustain no

injury whatever when intelligently subjected to an equally effective sterilization with steam. The same may be said of cotton and woollen fabrics, bedding, clothing, etc.

Aside from the relations of the two methods to the materials to be sterilized, their action toward the organisms to be destroyed is quite different. The penetrating power of steam renders it by far the more efficient agent of the two. The spores of several organisms which are killed by an exposure of but a few moments to the action of steam, resist the destructive action of dry heat at a higher temperature for a much greater length of time.

These differences will be strikingly brought out in the experimental work on this subject. For our purposes it is necessary to remember that the two methods have the following applications:

The dry method, at a temperature of 150°–180° C. for one hour, is employed for the sterilization of glassware such as flasks, test-tubes, culture-dishes, pipettes, plates, etc.

Sterilization by steam is practised with all culture-media, whether fluid or solid. Bouillon, milk, gelatin, agar-agar, potato, etc., are under no circumstances to be subjected to dry heat.

Discontinued Sterilization.—The manner in which heat is employed in processes of sterilization varies with circumstances. When used in the dry form its application is always continuous—*i. e.*, the objects to be sterilized are simply exposed to the proper temperature for the length of time necessary to destroy all living organisms which may be upon them. With the use of steam, on the other hand, the articles to be sterilized are frequently of such a nature that a prolonged application of heat might materially injure them. For this and other reasons steam is usually applied inter-

mittently and for short periods of time. The principles involved in the intermittent method of sterilization depend upon differences of resistance to heat which the organisms to be destroyed are known to possess at different stages of their development. During the life cycle of many of the bacilli there is a stage in which the resistance of the organism to the action of both chemical and thermal agents is much greater than at other stages of their development. This increased power of resistance appears when these organisms are in the *spore-* or *resting-stage*, to which reference has already been made. When in the vegetative or growing stage most bacteria are killed in a short time by a relatively low temperature; whereas, under conditions which favor the production of spores, the spores are seen to be capable of resisting very much higher temperatures for an appreciably longer time; indeed, spores of certain bacilli have been encountered that retain the power of germinating after an exposure of from five to six hours to the temperature of boiling water. Such powers of resistance have never been observed in the vegetative stage of development. These differences in resistance to heat which the spore-forming organisms possess at their different stages of development is taken advantage of in the process of sterilization by steam known as the *discontinuous*, *fractional*, or *intermittent method*, and are the essential feature of the principles on which the method is based.

As culture-media are dependent for their usefulness upon the presence of more or less unstable organic compounds, the object aimed at in this method is to destroy the organisms in the shortest time and with the least amount of heat. It is accomplished by subjecting them to the elevated temperature at a time when the bacteria are in the vegetat-

ing or growing stage—*i. e.*, the stage at which they are most susceptible to detrimental influences. In order to accomplish this it is necessary that there should exist conditions of temperature, nutrition, and moisture which favor the vegetation of the bacilli and the germination of any spores that may be present. When, as in freshly prepared nutrient media, this combination is found, the spore-forming organisms are not only less likely to enter the spore-stage than when their environment is less favorable to their vegetation, but spores which may already exist develop very quickly into mature cells.

It is plain, then, that with the first application of steam to the substance to be sterilized the mature vegetative forms are destroyed; while certain spores that may be present resist this treatment, providing the sterilization is not continued for too long a time. If now the sterilization be discontinued, and the material which presents conditions favorable to the germination of the spores be allowed to stand for a time, usually for about twenty-four hours, at a temperature of from 20° to 22° C., those spores which resisted the action of the steam will, in the course of this interval, germinate into the less resistant vegetative cells. A second short exposure to the steam kills these forms in turn, and by a repetition of this process all bacteria that were present may be destroyed without the application of the steam having been of long duration at any time. It should be remembered that while spores which may be present are not directly killed by such an exposure to heat as they experience in the intermittent method of sterilization, still their power of germination is somewhat inhibited by this treatment. In this method, therefore, if the temperature of 100° C. be employed for too long a time, it is

possible so to retard the germination of the spores as to render it impossible for them to develop into the vegetative stage during the interval between the heatings. By excessively long exposures to high temperature, but not long enough to destroy the spores directly, the object aimed at in the method may be defeated, and in the end the substance undergoing sterilization be found still to contain living bacteria. In this process the plan that has given most satisfactory results is to subject the materials to be sterilized to the action of steam, under the ordinary conditions of atmospheric pressure, for fifteen minutes on each of three successive days, and during the intervals to maintain them at a temperature of about 25°–30° C. At the end of this time all living organisms which were present will, as a general rule, have been destroyed, and, unless opportunity is given for the access of new organisms from without, the substances thus treated remain sterile. As an exception to this, certain species of spore-forming bacteria are occasionally encountered that are not readily destroyed by this mode of treatment. These species are found so uniformly in the soil that the customary designation for them is that of "the soil bacteria." This group includes a number of species that are endowed with remarkable resistance to heat. Some of them are probably thermophilic by nature, which would account not only for the failure to destroy their spores by the ordinary exposures to steam, but also for their slow and incomplete development from the spore to the less resistant vegetative stage during the intervals between the heatings, for, as a rule, the materials containing them are kept at a temperature during these intervals that is too low to favor the rapid germination of the species having thermophilic tendencies.

As a result of the presence of these species, media that

have been subjected to the customary discontinuous method of sterilization may, after having been kept for a time, reveal the presence of isolated colonies of bacteria distributed through them in such a way as to preclude all likelihood of their having fallen upon it from the air after sterilization was supposedly complete.

Theobald Smith¹ has called attention to an instructive personal experience. He finds that when media are present in vessels in only thin layers the spores of *anaërobic* species do not develop into the vegetative forms during the interval between the heatings, for the reason that the shallow layer of medium does not sufficiently exclude free oxygen to permit it; and by subjecting such materials, *apparently* sterilized by the intermittent method, to strictly anaërobic conditions a development of anaërobic species will often occur. On the other hand, if the vessels be nearly filled with media, and especially if the area of the surface be small, the conditions are much more favorable to the germination of anaërobic spores, and sterilization by this process after such precautions is usually perfect.

Fortunately, these undesirable experiences are rare, but that they do occur, and result in no small degree of annoyance, will be admitted by most bacteriologists.

It must be borne in mind that this method of sterilization is only applicable in those cases which present conditions favorable to the germination of the spores into mature vegetative cells. Dry substances, such as instruments, bandages, apparatus, etc., or organic materials in which decomposition is far advanced, where conditions of nutrition favorable to the germination of spores are not present, do not offer the conditions requisite for the successful operation

¹ Journal of Experimental Medicine, iii, No. 6, p. 647.

of the principles underlying the intermittent method of sterilization.

Discontinued Sterilization at Low Temperatures.—The process of discontinued sterilization at *low* temperatures is based upon exactly the same principle, but differs in two respects from the foregoing in the manner by which it is practised, viz., it requires a greater number of exposures for its accomplishment, and the temperature at which it is conducted is not above 68°–70° C. It is employed for the sterilization of easily decomposable materials, which would be rendered useless by steam, but which are unaltered by the temperature employed, and for certain albuminous culture-media that it is desirable to retain in a fluid condition during sterilization, but which would be coagulated if exposed to higher temperatures. This process requires that the material to be sterilized should be subjected to a temperature of 68°–70° C. for one hour on each of six successive days, the interval of twenty-four hours between the exposures admitting of the germination of spores into mature cells. During this interval the substances under treatment are kept at about 25°–30° C. The temperature employed in this process suffices to destroy, in about one hour, the vitality of almost all organisms in the vegetative stage. Formerly blood-serum was always sterilized by the intermittent method at a low temperature.

Direct Sterilization.—Sterilization by steam is also practised by what may be called the direct method—that is to say, both the mature organisms and the spores which may be present in the material to be sterilized are destroyed by a single exposure to the steam. In this method steam at its ordinary temperature and pressure—live steam or streaming steam, as it is called—is employed just as in the

first method described; but it is allowed to act for a much longer time, usually for not less than an hour; or steam under pressure, and consequently of a higher temperature, is now frequently employed. By the latter procedure a single exposure of fifteen minutes is sufficient for the destruction of practically all bacilli and their spores, providing the pressure of the steam is not less than one atmosphere over and above that of normal; this is approximately equivalent to a temperature of 122° C. to which the organisms are exposed.

The objection that has been urged to both of these methods, particularly that in which steam under pressure is employed, is that the properties of the media are altered. Gelatin is said to become cloudy and lose the property of solidifying; in bouillon and agar-agar fine precipitates are said to result, and some believe the reaction undergoes a change. In the experience of those who have used steam under pressure not exceeding one atmosphere for ten to fifteen minutes these obstacles have rarely been encountered. There is one point to be borne in mind, however, in using steam under pressure, viz., it is not possible to regulate the *time of exposure* to the same degree of nicety as where ordinary live steam is used. The reason for this is that if the apparatus be opened to remove the objects being sterilized while the steam within it is under pressure, the escape of steam will be so rapid that all fluids within the chamber, thus suddenly relieved of pressure, will begin to boil violently, and, as a rule, will boil quite out of the tubes, flasks, etc., containing them. For this reason the apparatus must be kept closed until cool, or until the gauge indicates that pressure no longer exists within the chamber, and even then it should be opened very cautiously. It is patent from

this that the temperature and time of exposure of articles sterilized by this process cannot usually be controlled with accuracy. It requires some time to reach a given pressure after the apparatus is closed, and it also requires time for cooling after the desired exposure to such pressure before the apparatus can be opened.

It is manifest that during these three periods, viz., (a) reaching the pressure desired, (b) time during which the pressure is maintained, and (c) time for fall of pressure before the chamber can be opened, it is difficult to say certainly to what temperature and pressure the articles in the apparatus have, *on the whole*, been subjected. Clearly, if the desired pressure and temperature have been maintained for ten minutes, one cannot say that that is *all* the heat to which the articles have been subjected during their stay in the chamber. In this light, while steam under pressure may answer very well for routine sterilization, still it presents insurmountable obstacles to its use in more delicate experiments where time-exposure to definite temperature is of importance. Nevertheless, for general laboratory purposes, sterilization by steam under pressure has so much to recommend it in the way of economy of time and certainty of accomplishment that it has practically superseded the older methods of sterilization by streaming or live steam; and in most laboratories the original styles of steam sterilizers are rapidly giving way to some one or another of the modern forms of autoclave.

For sterilization by live steam the apparatus in common use was for a long time the cylindrical boiler recommended by Koch. (See Fig. 8.) Its construction is very simple, essentially that of the ordinary domestic potato-steamer. It consists of a copper cylinder, the lower fifth, approximately,

of which is somewhat larger in circumference than the remaining four-fifths and serves as a reservoir for the water from which the steam is to be generated. Covering this section of the cylinder is a wire rack or grating, through which the steam passes, and which supports the articles to be sterilized. Above this, comprising the remaining four-

FIG. 8

Steam sterilizer, pattern of Koch.

fifths of the cylinder, is the chamber for the reception of the materials over and through which the steam is to pass. The cylinder is closed by a snugly fitting cover, through which are usually two perforations, into which a thermometer and a manometer may be inserted. The whole of the outer surface of the apparatus is encased in a non-conducting mantle of asbestos or felt.

The water is heated by a gas-flame placed in an enclosed chamber, upon which the apparatus rests, which serves to diminish the loss of heat and deflection of the flame through the action of draughts. The apparatus is simple in construction, and the only point which is to be observed while using it is the level of the water in the reservoir. On the reservoir is a water-gauge which indicates at all times the

FIG. 9

Arnold steam sterilizer.

amount of water in the apparatus. The amount of water should never be too small to be indicated by the gauge; otherwise there is danger of the reservoir becoming dry and the bottom of the apparatus being destroyed by the direct action of the flame.

A sterilizer that has come into very general use in bacteriological laboratories is one originally intended for use

in the kitchen. It is called the "Arnold steam sterilizer." It is very ingenious in its construction as well as economical in its employment.

The difference between this apparatus and that just described is that it provides for the condensation of the steam after its escape from the sterilizing chamber, and returns the water of condensation automatically to the reservoir, so that in practice the apparatus requires but little attention, as with ordinary care there is no likelihood of the water in the reservoir becoming exhausted, with the consequent destruction of the sterilizer. Fig. 9 shows a section through this apparatus.

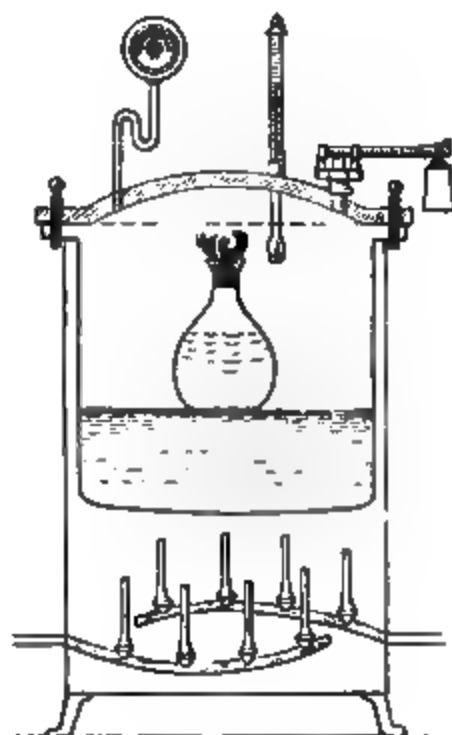
STERILIZATION UNDER PRESSURE.

The advantages of the use of steam under pressure for the purposes of sterilization have received such general recognition that almost everywhere this method is supplanting the older one of intermittent sterilization with streaming or live steam. By this plan one is able to accomplish, by a single exposure of fifteen minutes to steam under a pressure of one atmosphere, the same end that would, with streaming steam, require three exposures of fifteen minutes on each of three successive days.

For sterilization by steam *under pressure* several special forms of apparatus exist. The principles involved in them all are, however, the same. They provide for the generation of steam in a chamber from which it cannot escape when the apparatus is closed. Upon the cover of this chamber is a safety-valve, which can be regulated so that any degree of pressure (and coincidently of temperature) that is desirable may be maintained within the sterilizing chamber.

These sterilizers are known as "digesters" and as "autoclaves." Their construction can best be understood by reference to Figs. 10 and 11.

FIG. 10

*A**B*

Autoclave. *A*, external appearance; *B*, section.

STERILIZATION BY HOT AIR.

The hot-air sterilizers used in laboratories are simply double-walled boxes of Russian or Swedish iron (Fig. 12), having a double-walled door, which closes tightly, and a heavy copper bottom. They are provided with openings for the escape of the contained air and the entrance of the heated air. The flame, usually from a rose-burner (Fig. 13),

is applied directly to the bottom. The heat circulates from the lower surface around about the apparatus through the space between its walls.

FIG. 11

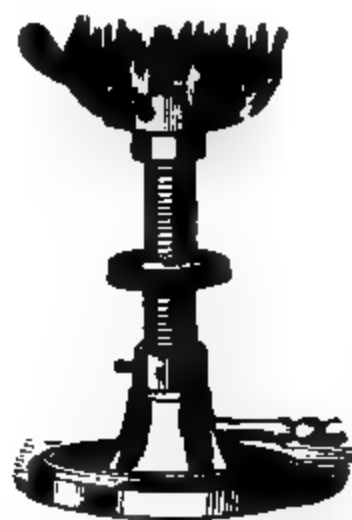
Autoclave or digester for sterilizing by steam under pressure.

The construction of the copper bottom of the apparatus upon which the flame impinges is designed to prevent the direct action of the flame upon the sheet-iron bottom of the chamber. It consists of several copper plates placed one

above the other, but with a space of about 4 to 5 mm. between the plates. These copper bottoms after a time become burned out, and unless they are replaced the apparatus is useless. The older forms of hot-air sterilizers are so constructed that their repair is a matter involving some time and expense. To meet this objection I had constructed

FIG. 12

FIG. 13



Laboratory hot-air sterilizer.

Rose-burner.

some years ago a sterilizer in all respects similar to the old form except in the arrangement of the copper bottom. This latter is made in such a way that it can easily be removed, so that by keeping several sets of copper plates on hand a new plate can readily be inserted when the old one is burned out.

In the employment of the hot-air sterilizer care should

always be given to the condition of the copper bottom; for the direct application of heat to the sheet-iron plate upon which the substances to be sterilized stand results not only in destruction of the apparatus, but frequently in destruction of the substances undergoing sterilization.

Since the temperature at which this form of sterilization is usually accomplished is high, from 150° to 180° C., it is well to have the apparatus encased in asbestos boards, to diminish the radiation of heat from its surfaces. This not only confines the heat to the apparatus, but guards against the destructive action of the radiated heat on woodwork, furniture, etc., that may be in the neighborhood.

Thermal Death-point of Bacteria.—By “thermal death-point of bacteria” is meant the temperature necessary to kill them in a given time. As this varies with different species, it is an aid to identification. For the practical purposes of the sanitarian the knowledge is of fundamental importance. The thermal death-point of an organism is ascertained by subjecting it to varying degrees of temperature for five or ten minutes until the point is reached where it is killed. The test is best carried out by means of small glass bulbs, the so-called Sternberg bulbs, or through the use of capillary tubes containing a small amount of fluid inoculated with the organism to be studied. The bulb, or tube, is sealed in the gas flame and placed in a water-bath kept at 50° C. for five minutes. Sub-cultures are now made to learn whether the bacteria have been killed or not. If the organism survives the test is repeated at 55°, 60°, 65°, and 70° C. Finally, the test is repeated for each degree of temperature between the points where growth is still apparent and where the organisms have been killed. If the bacteria were killed when heated to 60° C. for five minutes, but sur-

vived when heated to 55° C., then similar tests are made for the same length of time for each degree of temperature between 55° and 60° C. It will usually be found that heating for ten minutes suffices to kill the bacteria at a temperature one or two degrees lower than that required when heated for only five minutes. All such tests should be made at least in duplicate, and the mean of the results taken.

CHEMICAL STERILIZATION AND DISINFECTION.

As has been stated, it is possible by means of certain chemical substances to destroy all bacteria and their spores that may be within or upon various materials and objects—*i. e.*, to sterilize them; and it is also possible by the same means to rob objects of their dangerous infective properties without at the same time sterilizing them—*i. e.*, to disinfect them. This latter process depends upon the fact that the vitality of many of the less resistant *pathogenic* organisms is easily destroyed by an exposure to particular chemical substances that may be without effect upon the more resistant saprophytes and their spores that are present.

In general, the use of chemicals for sterilization is not to be considered in connection with substances that are to be employed as culture-media, and their employment is restricted in the laboratory to materials that are of no further value, and to infected articles that are not injured by the action of the agents used, though exceptionally such volatile germicides as chloroform and ether are employed for the sterilization of special culture-media. (See Preservation of Blood-serum with Chloroform.) In short, they are mainly of value in rendering infected waste-material innocuous. For the successful performance of this form of disinfection

there is one fundamental rule always to be borne in mind, viz., it is essential to success that the disinfectant used should come in direct contact with the bacteria to be destroyed, otherwise there is no disinfection.

For this reason one should always remember, in selecting the disinfecting agent, the nature of the materials containing the bacteria upon which it is to act, for the majority of disinfectants, and particularly those of an inorganic nature, vary in the degree of their potency with the chemical nature of the mass to which they are applied. Often the materials containing the bacteria to be destroyed are of such a character that they combine with the disinfecting agent to form insoluble, more or less inert precipitates; these so interfere with the penetration of the disinfectant that many bacteria may escape its destructive action entirely and no disinfection be accomplished, although an agent may have been employed that would, under other circumstances, have given entirely satisfactory results.

An *antiseptic* is a body which, by its presence, prevents the growth of bacteria without of necessity killing them. A body may be an antiseptic without possessing disinfecting properties to any very high degree, but a disinfectant is always an antiseptic as well.

A *germicide* is a body possessing the property of killing bacteria.

Mode of Action of Disinfectants.—In the destruction of bacteria by means of chemical substances there occurs, most probably, a definite chemical reaction—that is to say, the characteristics both of the bacteria and the agent employed in their destruction are lost in the production of an inert third body, the result of their combination. It is impossible to state with certainty, as yet, that this is in

general the case; but the evidence that is rapidly accruing from studies upon disinfectants and their mode of action points strongly to the accuracy of this belief. This reaction, in which the typical structures of both bodies concerned are lost, takes place between the agent employed for disinfection and the protoplasm of the bacteria. For example, in the reaction that is seen to take place between the salts of mercury and albuminous bodies there results a third compound, which has neither all the characteristics of mercury nor of albumin, but partakes of some of the peculiarities of both; it is a combination of albumin and mercury, commonly known by the indefinite term "albuminate of mercury." Some such reaction as this apparently occurs when the soluble salts of mercury are brought in contact with bacteria. This view has been strengthened by the experiments of Geppert, in which the reaction was caused to take place between the spores of the anthrax bacillus and a solution of mercuric chloride, the result being the *apparent* destruction of the vitality of the spores by the formation of this third, inert compound. In these experiments it was shown that though this combination had taken place, still it did not of necessity imply the death of the spores, for if by proper means the combination of mercury with their protoplasm was broken up, many of the spores resumed their vitality, with all their previous disease-producing and cultural peculiarities. Geppert employed a solution of ammonium sulphide for the purpose of destroying the combination of spore-protoplasm and mercury; the mercury was precipitated from the protoplasm as an insoluble sulphide, and the protoplasm of the spores returned to its original condition. These and other somewhat similar experiments have given a new impulse to the study of disinfectants, and

in the light shed by them many of our previously formed ideas concerning the action of disinfecting agents have been modified.

The process of disinfection is not a catalytic one—*i. e.*, occurring simply as a result of the presence of the disinfecting body, which is not itself decomposed during its process of destruction—but is, as said, a definite chemical reaction occurring within more or less fixed limits; that is to say, with a given amount of the disinfectant just so much work, expressed in terms of destruction of bacteria can be accomplished.

Another point in favor of this view is the increased energy of the reaction with elevation of temperature. Just as in other chemical phenomena the intensity and rapidity of the reaction become greater under the influence of heat, so in the process of disinfection the combination between the disinfectant and the organisms to be destroyed is much more energetic at a temperature of 37°–39° C. than it is at 12°–15° C.

A number of important and novel suggestions with regard to the *modus operandi* of disinfection were brought out through the work of Krönig and Paul,¹ who took up the subject from its physico-chemical standpoint. The comprehensive nature of this elaborate investigation precludes more than a brief mention of some of the conclusions reached, and in order that these may be intelligible, certain beliefs (working hypotheses) of the physical chemists should be borne in mind. In 1887 Arrhenius proposed the theory that when an electrolyte (a compound decomposable by an electric current) is dissolved in water its molecules break down, not simply into their component atoms, but into

¹ *Zeitschrift für Hygiene und Infektionskrankheiten*, 1897, xxv, 1–112.

ions, which are atoms or groups of atoms having electro-positive and electro-negative characteristics. According to this theory, salts, when dissolved in water, undergo electrolytic dissociation into metallic and acidic *ions*, the former being the electro-positive *cation*, the latter the electro-negative *anion*; sodium chloride, for example, resolving itself, under these conditions, into its sodium, or *metal ion*, and its chlorine, or *acidic ion*. The electro-positive cations, according to Ostwald, comprise the metals and metal-like radicals, such as ammonium (NH_4) and hydrogen (H); while the electronegative anions include the halogens, the acidic radicals (such as NO_3 and SO_4), and hydrosyl.¹ Using this theory as the basis of their investigations, Krönig and Paul reached the following conclusions with regard to the action of chemical disinfectants:

The germicidal value of a metallic salt depends not only upon its specific character, but also upon that of its *anion*.

Solutions of metallic salts in which the metallic part is represented by a complex ion and in which the concentration of the metal ion is very slight, have but feeble disinfecting activity.

The halogen compounds of mercury act according to the degree of their dissociation.

The disinfecting power of the halogens—chlorine, bromine, iodine—(as well as their compounds) is in inverse ratio to their atomic weights.

The disinfecting activity of watery solutions of mercuric chloride is diminished by the addition to them of other

¹ Consult Ostwald's *Lehrbuch der Allg. Chemie*; or Muir's translation of Ostwald's *Solutions*, p. 189, published by Longmans, Green & Co., London and New York, 1891. Also *The Rise of the Theory of Electrolytic Dissociation*, etc., by H. C. Jones, Ph.D., Johns Hopkins Hospital Bulletin, No. 87, June, 1898, p. 136.

halogen compounds of metals and of hydrochloric acid. It appears probable that this is due to obstruction offered to electrolytic dissociation.

The disinfecting activities of watery solutions of mercuric nitrate, mercuric sulphate, and mercuric acetate are increased by the moderate addition of sodium chloride.

In general, acids disinfect according to the degree of their dissociation—*i. e.*, according to the concentration of their hydrogen ions in the solution.

The bases, potassium, sodium, lithium, and ammonium hydroxide, disinfect according to the degree of their dissociation—*i. e.*, corresponding to the concentration of their hydroxyl ions in the solution.

The disinfecting activity of metallic salts is, in general, less in albuminous fluids than in water. It is probable that this is due to a diminution in the concentration of metallic ions in the solution.

The reaction between the inorganic salts and albuminous bodies is not selective; they combine in most instances with any or all protoplasmic bodies present. For this reason the employment of many of the commoner disinfectants in general practice is a matter of doubtful advantage. For example, the disinfection of excreta, sputum, or blood, containing pathogenic organisms, by means of corrosive sublimate, is a procedure of questionable success. The amount of sublimate employed may be entirely used up and rendered inactive as a disinfectant by the ordinary protoplasmic substances present, without having any appreciable effect upon the bacteria which may be in the mass.

These remarks are introduced in order to guard against the implicit confidence so often placed in the disinfecting

value of corrosive sublimate. In many bacteriological laboratories it is the custom to keep at hand vessels containing solutions of corrosive sublimate, into which infectious materials may be placed. The value of this procedure, as we have just learned, may be more or less questionable, especially in those cases in which the substance to be disinfected is of a proteid nature and where the solution used is not freshly prepared and frequently replenished. On the introduction of such substances into the sublimate solution the mercury is quickly precipitated by the albumin, and its disinfecting properties may be in large part or entirely destroyed; we may in a very short time have little else than water containing an inactive precipitate of albumin and mercury, in so far as its value as a disinfectant is concerned.

Though the other inorganic salts have not been so thoroughly studied in this connection, it is nevertheless probable that the same precautions should be taken in their employment as we now know to be necessary in the use of the salts of mercury.

The modes of action of other germicides have not been so carefully investigated as has that of the metallic salts. From the nature of many of them, however, we may infer that some act through oxidation, as in the case of strong acids and other active oxidizers; others by coagulation or by dehydration, as in the case of strong aldehydes and alcohols; and others by penetrating the cell wall and fatally poisoning the bacterial protoplasm, as in the case of hydrocyanic acid and its compounds.

Practical Disinfection.—Where it is desirable to use chemical disinfectants in the laboratory, much more satisfactory results can usually be obtained from the employment of

carbolic acid in solution. A 3 or 4 per cent. solution of commercial carbolic acid in water requires longer for disinfection; but it is, at the same time, open to fewer objections than are solutions of the inorganic salts; though here, too, we find a somewhat analogous reaction between the carbolic acid and proteid matters. Under ordinary circumstances its action is complete in from twenty minutes to a half-hour. It is not reliable for the disinfection of resistant spores; such, for instance, as those of *bacillus anthracis*.

All tissues containing infectious organisms should be *burned*, and all cloths, test-tubes, flasks, and dishes should be boiled in 2 per cent. soda (ordinary washing-soda) solution for fifteen to twenty minutes, or placed in the steam sterilizer for half an hour.

Intestinal evacuations may best be disinfected with boiling water or with *milk of lime*, a mixture composed of lime in solution and in suspension—ordinary fluid “white wash.” This should be thoroughly mixed with the evacuations until the mass contains a considerable excess of the lime, and should remain in contact with them for one or two hours. Excreta may also be easily disinfected by thoroughly mixing them with two or three times their volume of *boiling* water, after which they are kept covered until cool.

Sputum in which tubercle bacilli are present, as well as the vessel containing it, must be boiled in 2 per cent. soda for fifteen minutes, or steamed in the sterilizer for at least a half-hour.

On the whole, in the laboratory we should rely more upon the destructive properties of heat than upon those of chemical agents.

From what has been said, the absurdity of sprinkling here and there a little carbolic acid, or of placing vessels of carbolic acid about apartments in which infectious diseases are in progress, must be plain. Treatment of water-closets and cesspools by allowing now and then a few cubic centimeters of some so-called disinfectant to trickle through the pipes is ridiculous. A disinfectant must be *applied to the bacteria, and must be in contact with them for a long enough time to insure the destruction of their life.*

In the light of the latest experiments upon disinfectants, the place formerly occupied by many agents in the list of substances employed for the purpose will most likely be changed as they are studied more closely. The agents, then, which will prove of greatest value in the laboratory for the purpose of rendering infectious materials harmless are: *heat*, either by burning, by steaming for from half an hour to an hour, or by boiling in a 2 per cent. sodium carbonate solution for fifteen minutes; 3 to 4 per cent. solution of commercial carbolic acid; *milk of lime*, and a solution of *chlorinated lime* ("chloride of lime") containing not less than 0.25 per cent. of free chlorine. The chloride of lime from which such a solution is to be made should be fresh and of good quality. Good chlorinated lime, as purchased in the shops, should contain not less than 25 to 30 per cent. of available chlorine. The materials to be disinfected in either of the lime solutions should remain in them for about two hours. The solutions should be freshly prepared when needed, as they rapidly decompose upon standing.

CHAPTER IV.

Principles Involved in the Methods of Isolation of Bacteria in Pure Culture by the Plate Method of Koch—Materials Employed.

As was stated in the introductory chapter, the isolation in pure cultures of the different species that may be present in mixtures of bacteria was rendered possible only through the methods suggested by Koch. Since the adoption of these methods they have undergone many modifications, but the fundamental principle remains the same. The observation that lead to their development is of almost daily occurrence. When bread, cooked potatoes or old bits of leather are left in moist, damp surroundings they invariably become "moldy" as we call it; that is to say, they become more or less covered or spotted with deposits that are known to be composed of living micro-organisms.

If one watches the evolution of this condition from day to day it will be seen that the moldy deposit begins as a number of small isolated points which, as they get larger, may finally coalesce into a confluent mass that eventually covers the surface. If one examine these points, however, before they begin to run together, it is found that they are composed of micro-organisms of several different kinds, some being molds, some yeasts, and some bacteria. The isolated growths of these various species present different naked-eye appearances, so that even at a glance we are justified in suspecting that they are of a different nature. They develop from single cells that have fallen upon the

moist objects from the air, and as the cell grows and multiplies it forms these circumscribed patches or "colonies" as they are called.

The question that then presented itself was: If from a mixture of organisms floating in the air it is possible in this way to obtain in pure cultures the component individuals, what means can be employed for obtaining the same results at *will* from mixture of different species of bacteria when found together under other conditions? It was plain that the organisms were to be distinguished primarily, the one from the other, only by the structure and general appearance of the colonies growing from them, for by their morphology alone this is impossible. What means might be devised, then, for separating the individual members of a mixture in such a way that they would remain in a fixed position, and be so widely separated, the one from the other, as not to interfere with the production of colonies of characteristic appearance, which would, under favorable conditions, develop from each individual cell?

If one take in the hand a mixture of several kinds of flower seeds and attempt to separate the mass into its constituents by picking out the different grains, the task is tedious, to say the least of it; but if the handful of seeds be thrown upon a large flat surface, as upon a table, the grains become widely separated and the matter is considerably simplified; or, if sown upon proper soil, the various grains germinate and develop into plants of entirely different characteristics, by which they can readily be recognized as distinct species. Similarly, if a test-tube of decomposed bouillon be poured upon a large, flat surface, the individual bacteria in the mass are much more widely separated, the one from the other, than they were when the bouillon was

in the tube; but they are in a fluid medium; and there is no possibility of their either remaining separated or of their colonizing under these conditions, so that it is impossible by this means to pick out the individuals from the mixture.

FIG. 14

Showing certain macroscopic characteristics of colonies. Natural size.

If, however, some substance can be found which possesses the property of being at one time fluid and at another time solid, and which can be added to this bouillon without in any way interfering with the life-functions of the bacteria, then, as solidification set in, the organisms would be fixed

in their positions, and the conditions would be analogous to those seen on the bits of potato, bread or leather.

Gelatin possesses this property, and it was, therefore, used. At a temperature which does not interfere with the life of the bacteria it is quite fluid, whereas when subjected to a lower temperature it solidifies. When once solid it may be kept at a temperature favorable to the growth of the bacteria and will remain in its solid state.

Gelatin was added to the fluids containing mixtures of bacteria, and the whole was then poured upon a large, flat surface, allowed to solidify, and the results noted. It was found that the conditions seen on the slice of moldy potato could be reproduced; that the individuals in the mixture of bacteria grew well in the gelatin, and, as on the potato, grew in colonies of typical macroscopic peculiarities, so that they could easily be distinguished the one from the other by their naked-eye appearances. (See Fig. 14.) It was necessary, however, to use a more dilute mixture of bacteria than the original decomposed bouillon. The number of individuals in the tube was so enormous that on the gelatin plate they were so closely packed together that it was impossible to pick them out, not only because of their proximity the one to the other, but also because this packing together materially interfered with the production of those characteristic differences visible to the naked eye. The numbers of the organisms were then diminished by a process of dilution, consisting of transferring a small portion of the original mixture into a second tube of sterilized bouillon to which gelatin had been added and liquefied; from this a portion was added to a third gelatin-bouillon tube, and so on. These were then poured upon large, cold surfaces and allowed to solidify. The result was entirely satisfactory.

On the gelatin plates from the original tube, as was expected, the colonies were too numerous to be of use; on the plates made from the first dilution they were much fewer in number, but usually they were still too numerous and too closely packed to permit of characteristic growth; on the second dilution they were, as a rule, fewer in number and widely separated, so that the individuals of each species were in no way prevented by the proximity of their neighbors from growing each in its typical way. (See Fig. 15.) There

FIG. 15

*A**B**C*

Series of plates showing the results of dilution upon the number of colonies: *A*, Plate No. 1, or "original;" *B*, first dilution, or Plate No. 2; *C*, second dilution, or Plate No. 3. About one-fourth natural size.

was then no difficulty in picking out the colonies resulting from the growth of the different individual bacteria. This, then, is the principle underlying Koch's method for the isolation of bacteria by means of solid media.

The fundamental constituent of the media employed is the bouillon, which contains all the elements necessary for the nutrition of most bacteria, the gelatin being employed simply for the purpose of rendering the bouillon solid. The medium on which the organisms are growing is, therefore, simply solidified bouillon, or beef-tea.

In practice two gelatinous substances are employed—the one an animal or bone gelatin, the ordinary table gelatin of good quality; the other a vegetable gum, known as agar-agar, the native name for Ceylon moss or Bengal isinglass, which is obtained from a group of marine algæ found along the coast of Japan, China, and many parts of the East, where it is employed as an article of diet by the natives.

The behavior of the two gelatinous substances under the influence of heat and of bacterial growth renders them of different application in bacteriological work. The animal gelatin liquefies at a much lower temperature, and also requires a lower temperature for its solidification, than does the agar-agar. Ordinary gelatin, in the proportion commonly used in this work, liquefies at about 24° – 26° C., and becomes solid at from 8° – 10° C. It may be employed for those organisms which do not require a higher temperature for their development than 22° – 24° C. Agar-agar, on the other hand, does not liquefy until the temperature has reached about 98° – 99° C. It remains fluid ordinarily until the temperature has fallen to 38° – 39° C., when it rapidly solidifies. For our purposes, only that form of agar-agar can be used which remains fluid at from 38° – 40° C. Agar-agar which remains fluid only at a temperature above this point would be too hot, when in a fluid state, for use; many of the organisms introduced into it would either be destroyed or checked in their development by so high a temperature. Agar-agar is employed in those cases in which the cultivation must be conducted at a temperature above the melting-point of gelatin.

In addition to their thermal reactions, these two gelatinous substances are affected very differently by different

species of bacteria. As was said above, and as we shall soon see for ourselves, certain bacteria elaborate in the course of their growth digestive enzymes or ferments that in their action upon proteid matters are strikingly like pepsin in some and trypsin in other instances. When bacteria endowed with this physiological property are cultivated upon bone gelatin their growth is accompanied by the progressive digestion (liquefaction) of the gelatin, which liquefied gelatin cannot again be brought to a solid condition. We know of no bacteria capable of producing a similar liquefaction of agar-agar or vegetable gum.

As a rule, the colony-formations seen upon gelatin are much more characteristic than those which develop on agar-agar, and for this reason gelatin is to be preferred when circumstances will permit. Both gelatin and agar-agar may be used for the isolation of species from mixtures.

CHAPTER V.

Preparation of Media—Bouillon, Gelatin, Agar-agar, Potato, Blood-serum, Blood-serum from Small Animals, Milk, Litmus-whey Milk, Durham's Peptone Solution, Lactose Litmus-agar, Löffler's Blood-serum Mixture, the Serum-water Media of Hiss, Guarnieri's Gelatin-agar Mixture.

As has been stated, the fundamental constituent of culture-media is beef-tea, or bouillon.

BOUILLON.

The directions of Koch for the preparation of this medium have undergone many modifications to meet special cases; but for general use the formula now employed is as follows: 500 grams of finely chopped lean beef, free from fat and tendons, are to be soaked in 1 liter of water for twenty-four hours, during which time the mixture is to remain in an ice-chest or to be otherwise kept at a low temperature. At the end of twenty-four hours it is to be strained through a coarse towel and pressed until a litre of fluid is obtained. To this are to be added 10 grams (1 per cent.) of dried peptone and 5 grams (0.5 per cent.) of common salt (NaCl). It is then to be rendered exactly neutral or very slightly alkaline to litmus paper with a few drops of a 4 per cent. sodium hydroxide solution. The mixture is then placed in an agate ware or porcelain-lined saucepan over a free flame, and kept at the boiling-point until all the albumin is coagulated and the fluid portion is of a clear, pale straw color.

It is then filtered through a folded paper filter and sterilized by steam. Certain modifications of this method are of sufficient value to justify mention. Most important is the neutralization.

In the exhaustive paper of Fuller¹ on the question of reaction it was shown that the results obtained by titrating the same culture-medium with the same alkaline solution differed very markedly with the indicator employed. For instance, 1 liter of ordinary meat-infusion nutrient agar-agar required 47 c.c. of a normal caustic alkali solution to neutralize it when *phenolphthalein* was the indicator used, 28 c.c. when *blue litmus* was employed, and 5 c.c. when *rosolic acid* was substituted. It is manifest from this that the actual reactions of media, in the neutralization of which different indicators have been used, may differ very widely from one another, and that the results of cultivation on a medium neutralized by one method are not fairly comparable with those obtained when another indicator has been used. For the sake of uniformity Fuller suggests that bacteriologists should agree upon some one trustworthy method of neutralization and employ it to the exclusion of other methods. He recommends, as the procedure that has given the most satisfactory results in his hands, a modification of Schultz's method, viz., 5 c.c. of the culture-medium are to be mixed with 45 c.c. of distilled water in a porcelain evaporating-dish and boiled for three minutes, after which 1 c.c. of phenolphthalein solution² is added and titration with the one-twentieth normal caustic alkali solution is quickly made. The neutral point (slightly on the side of alkalinity)

¹ On the Proper Reaction of Nutrient Media for Bacterial Cultivation, Public Health (Journal of the American Public Health Association), Quarterly Series, 1895, vol. i, p. 381.

² A 0.5 per cent. solution of the powder in 50 per cent. alcohol.

is indicated by the appearance of a pink color, the effect of the alkali on the phenolphthalein. From the amount of one-twentieth normal alkali solution needed for 5 c.c. of the medium it is easy to calculate the number of cubic centimeters of the *normal* solution that will be required to neutralize the entire mass.

The phenolphthalein neutral point lies so high, averaging 47 c.c. of normal caustic alkali solution per liter for nutrient meat-infusion agar-agar, and 56 c.c. per liter for nutrient gelatin, that it is improbable from experience gained from the older methods that the conditions offered by media *neutral* to this indicator are suitable for the growth of all bacteria, so that with particular species it may be necessary to determine by experiment the degree of deviation from the neutral point that is best suited for development. In Fuller's experience the degree of deviation from the phenolphthalein neutral point that gives in general the best results is represented by from 15 to 20 of his scale—*i. e.*, there should remain enough uncombined acid in a liter of the finished medium to require the *further* addition of caustic alkali to the extent of from 15 to 20 c.c. of a normal solution to bring the reaction of the mass up to the phenolphthalein neutral point. Thus, for example, if upon titration it should be found that to *neutralize* a liter of nutrient meat-infusion gelatin by the phenolphthalein process 55 c.c. of normal caustic alkali solution would be needed, the *amount actually added* would be from 35 to 40 c.c.—*i. e.*, from 15 to 20 c.c. less than the amount needed to bring the reaction up to the neutral point.

Not infrequently the filtered bouillon, neutralized and sterilized, will be seen to contain a fine, flocculent precipi-

tate. This may be due either to excess of alkalinity or to incomplete precipitation of the albumin. The former may be corrected with dilute acetic or hydrochloric acid, and the bouillon again boiled, filtered, and sterilized; or, if due to the latter cause, subsequent boiling and filtration usually result in ridding the bouillon of the precipitate.

Another modification now generally employed is in the substitution of meat-extracts for chopped meat in making the bouillon. Almost any of the meat-extracts of commerce answer the purpose, though we usually employ Liebig's. It is used in the strength of from two to four grams to the litre of water. Peptone and sodium chloride are added as in the bouillon made from meat-infusion. The advantages of meat-extract are: it takes less time; affords a solution of more uniform composition if used in fixed proportions; and in general use gives results that are equally as satisfactory as those obtained from the employment of infusion of meat. The disadvantage is the possible presence of antiseptics or preservatives.

NUTRIENT GELATIN.

For the preparation of gelatin the bouillon is first made in the way given, except that its reaction is corrected *after* the gelatin has been completely dissolved, which occurs very rapidly in hot bouillon. The reaction of the gelatin of commerce is frequently more or less acid, so that a much larger amount of alkali is needed for its neutralization than for other media. It is possible, however, to obtain from the makers an excellent grade of gelatin from which practically all free acid has been carefully washed. The gelatin is

added in the proportion of 10 to 12 per cent. Its complete solution may be accomplished either over a water-bath, in the steam sterilizer, or over a free flame. If the latter method be practised, care must be taken that the mixture is constantly stirred to prevent burning at the bottom.

It is now almost the universal practice to use enamelled iron saucepans, instead of glass vessels for the purpose of making both gelatin and agar-agar; by this means the free flame may be employed without danger of breaking the vessel, and, with a little care, without burning the media. Under any conditions it is better to protect the bottom of the vessel from the direct action of the flame by the interposition of several layers of wire gauze, a thin sheet of asbestos-board, or an ordinary cast-iron stove-plate.

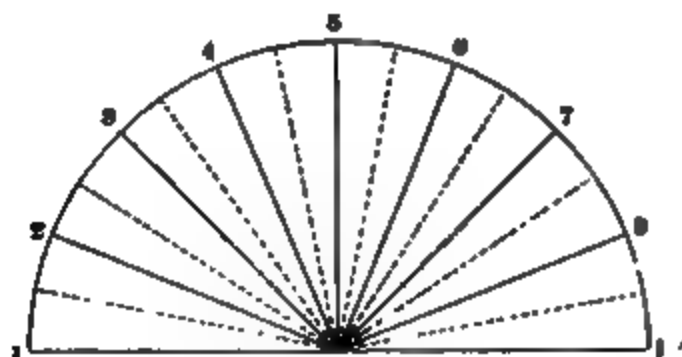
When the gelatin is completely melted it may be filtered through a folded paper filter supported on an ordinary funnel; if solution is complete, this should be very quickly accomplished.

To Fold a Filter.—For the filtration of such substances as gelatin and agar-agar it is of importance to have a properly folded filter. Inability to fold a filter properly is so common with beginners that a detailed description of the steps may not be out of place. To fold a filter correctly, proceed as follows: A circular piece of filter paper is folded exactly through its centre, forming the fold 1, 1' (Fig. 16); the end 1 is then folded over to 1', forming the fold 5; 1 and 1' are each then brought to 5, thus forming the folds 3 and 7; 1 is then carried to the point 7, and the fold 4 is formed, and by carrying 1' to 3 the fold 6 is produced; and by bringing 1 to 3 and 1' to 7 the folds 2 and 8 result.

Thus far the ridges of all folds are on the side of the paper

next to the table on which we are folding. The paper is now taken up and each space between the seams just produced is to be subdivided by a crease or fold through its centre, as indicated by the dotted lines in Fig. 16, but with

FIG. 16



the creases on the side *opposite* to that occupied by creases 1, 2, 3, 4, etc., first made. As each of these folds is made the paper is gradually brought into a wedge-shaped bundle (Fig. 17, *a*); which when opened assumes the form of a

FIG. 17



properly folded filter (seen in *b*, Fig. 17). Before placing it upon the funnel it is well to go over each crease and see that it is as closely folded as possible, care being taken not to tear it. The advantage of the folded filter is that by its

use a much greater filtering surface is obtained, as it is in contact with the funnel only at the points formed by the ridges, leaving the greater part of the flat surface free for filtration.

The employment of the hot-water funnel, so often recommended, has been dispensed with in this work to a very large extent, for the reason that if solution of the gelatin is complete, filtration is so rapid as not to necessitate the use of an apparatus for maintaining a high temperature. The temperature at which the hot-water funnel retains the gelatin is so high that evaporation and concentration rapidly occur, and in consequence filtration is, as a rule, retarded. The filtration is frequently done in the steam sterilizer; but this, too, is unnecessary if the gelatin is quite dissolved. At the ordinary temperature of the room, and by the means commonly employed for the filtration of other substances, both gelatin and agar-agar may be rapidly filtered if they are completely dissolved.

It not infrequently occurs that, even under the most careful treatment, the filtered gelatin is not quite transparent, and clarification becomes necessary. For this purpose the mass must be redissolved, and when at a temperature between 60° and 70° C. an egg, which has been beaten up with about 50 c.c. of water, is added. The whole is then thoroughly mixed together and again brought to the boiling-point, and kept there until coagulation of the albumin occurs. The egg albumin coagulates as large flocculent masses, and it is better not to break them up, as when broken up into fine flakes they clog the filter and materially retard filtration.

The practice sometimes recommended of removing these albuminous coagula by first filtering the gelatin through a

cloth, and then through paper, is not only superfluous, but in most instances renders the process of filtration much more difficult, because of the disintegration of the masses into finer particles, which have the effect just mentioned, viz., of clogging the filter.

Under no circumstances should a filter be used without first having been moistened with water. If this is not done, the pores of the paper, which are relatively large when in a dry state, when moistened by the gelatin not only diminish in size, but in contracting are often entirely occluded by the finer albuminous flakes which become fixed within them, and filtration practically ceases. The preliminary moistening with water causes diminution of the size of the pores to such an extent that the finer particles of the precipitate *rest on* the surface of the paper, instead of becoming fixed *in its meshes*.

During boiling it is well to filter, from time to time, a few cubic centimeters of the gelatin into a test-tube and boil it over a free flame for a minute or so; in this way one can detect if all the albumin has been coagulated—*i. e.*, if the solution is ready for filtration.

Gelatin should not, as a rule, be boiled more than ten or fifteen minutes at one time, or be left in the steam sterilizer for more than thirty minutes; otherwise its property of solidifying may be impaired.

As soon as the preparation of the gelatin is complete, whether it is retained in the flask into which it has been filtered or decanted into sterilized test-tubes, it should be sterilized, the mouth of the flask or the test-tubes containing it having been previously closed with cotton plugs. It may be sterilized by either the intermittent method with streaming steam or by a single application of steam under pressure

in the autoclave. If the latter method be selected, the pressure should not exceed one atmosphere and the time of exposure be not over fifteen minutes.

NUTRIENT AGAR-AGAR.

The preparation of nutrient agar-agar by the beginner is far too frequently a tedious and time-consuming operation. This is due mainly to lack of patience and to deviation from the rules laid down for the preparation of this medium. If the directions given below for the preparation of nutrient agar-agar be strictly observed, no difficulty whatever should be encountered. Many methods are recommended for its preparation, almost every worker having some slight modification of his own.

The methods that have given us the best results, and from which we have no good grounds for departing, are as follows:

Prepare the bouillon in the usual way. Agar-agar reacts neutral or very slightly alkaline, so that the bouillon may be neutralized before the agar-agar is added. Then add finely chopped or powdered agar-agar in the proportion of 1 to 1.5 per cent. Place the mixture in a porcelain-lined iron vessel, and on the side of the vessel make a mark at the height at which the level of the fluid stands; if a liter of medium is being made, add about 250 to 300 c.c. more of water and allow the mass to boil slowly, occasionally stirring, over a free flame, from one and a half to two hours; or until the excess of water—*i. e.*, the 250 or 300 c.c. that were added—has evaporated. Care must be taken that the mixture does not boil over the sides of the vessel. From time to time observe if the fluid has fallen below its original level; if it has, add hot water until its volume of 1 liter is

restored. At the end of the time given remove the flame and place the vessel containing the mixture in a large dish of cold water; stir the agar-agar continuously until it has cooled to about 68° – 70° C., and then add the white of one egg which has been beaten up on about 50 c.c. of water; or the ordinary dried albumin of commerce may be dissolved in cold water in the proportion of about 10 per cent. and used; the results are equally as good as when eggs are employed. Mix this carefully throughout the agar-agar and allow the mass to boil slowly for about another half-hour, observing all the while the level of the fluid, which should not fall below the liter mark. It is necessary to reduce the temperature of the mass to the point given, 68° – 70° C., otherwise the coagulation of the albumin will occur suddenly in lumps and masses as soon as it is added, and its clarifying action will not be uniform. The process of clarification with the egg is purely mechanical; the finer particles, which would otherwise pass through the pores of the filter, being taken up by the albumin as it coagulates and retained in the coagula.

At the end of a half-hour the boiling mass may be easily and quickly filtered through a heavy, folded paper filter *at the room temperature*; as a rule the filtrate is as clear and transparent as agar-agar usually appears.

It may be well to emphasize the fact that for the filtration of agar-agar no special device for maintaining the temperature of the mass, is necessary. Agar-agar prepared after the methods just given should pass through a properly folded paper filter at the rate of a litre in from twelve to fifteen minutes.

Another plan that insures complete solution of the agar-agar without causing the precipitates often seen when all

the ingredients are added at once and boiled for a long time is to weigh out the necessary amount of agar-agar, 10 or 15 grams, and place this in 1300 or 1400 c.c. of water and boil down over a free flame to 1000 c.c. The peptone, salt, and beef-extract are then added and the boiling continued until they are dissolved. The clarification with egg-albumen may then be done, and usually the mass filters quite clear and does not show the presence of precipitates upon cooling. If the mixture is positively alkaline, it is not only cloudy, but it filters with difficulty; if it is acid, it is usually quite clear, and filters more quickly, but, as Schultz has pointed out, it loses at the same time some of its gelatinizing properties.

Another method by which agar-agar can be easily and quickly melted is by steam under pressure. If the flask containing the mixture of bouillon and agar-agar be kept in the digester or autoclave for ten minutes with the steam under a pressure of about one atmosphere, as shown by the gauge, the agar-agar will be found at the end of this time completely melted, and filtration may then be accomplished with but little difficulty.

If glycerin is to be added to the agar-agar, it is done after filtration and before sterilization. The nutritive properties of the media for certain organisms, particularly the tubercle bacillus, are increased by the addition of glycerin in the proportion of 5 to 7 per cent.

If after filtration a fine flocculent precipitate is seen, look to the reaction of the medium. If it is quite alkaline, boil, neutralize, and filter again. If the reaction is neutral or only very slightly acid, dissolve and again clarify with egg-albumen by the method given.

The most important feature of all the media, aside from

the correct proportion of the ingredients, is their reaction. It must be neutral or very slightly alkaline to litmus. (See remarks on Neutralization of Media.) Only a few organisms develop well on media of an acid reaction.

PREPARATION OF POTATOES.

With an ordinary cork borer punch out from sound potatoes cylindrical bits that will slip easily into the test-tubes to be used. Cut away all particles of the skin. Then cut on each cylinder a slanting surface extending from about the middle diagonally to the end. Leave the cylinders in running water over night to prevent them from becoming discolored when they are sterilized.

FIG. 18

One potato cylinder thus prepared is then to be placed in each of the already cleaned, plugged and sterilized test-tubes, after which they are sterilized by either the intermittent method with streaming steam or by steam under pressure in the autoclave. In the latter event one atmosphere of pressure should be continued for twenty minutes. (See Fig. 18.)

For some purposes potatoes may be advantageously peeled, sliced into disks of about 1 cm. in thickness, and placed in small glass dishes provided with covers, similar to the ordinary crystallizing dishes. The dish and its contents are then sterilized by steam in the usual way. By this plan a relatively large area for cultivation is obtained.

Potato in test-tube.

Potatoes may also be boiled, or steamed, and mashed, and the mass placed in covered dishes, test-tubes, or flasks, and sterilized. By this method one obtains in the mass a mean of the composition of the several potatoes, or bits of potatoes, used in making it, an advantage where uniformity is desired.

Care must be given to the sterilization of potatoes, because they always have adhering to them the organisms commonly found in the ground, the spores of which are among the most resistant known.

BLOOD-SERUM.

For ordinary routine work blood-serum may be obtained from either the slaughter houses or the antitoxin manufacturers. When from the former the blood that streams from the severed vessels of the throat of the slaughtered animal is collected under as cleanly conditions as possible in large, clean glass museum jars. These are then, with the covers placed upon them, set aside in an ice-chest until coagulation is complete. The serum may then be decanted or pipetted off into flasks and thus transported to the laboratory to be sterilized by the method given below.

In many localities it is possible to purchase at a small cost normal horse serum in bulk from firms engaged in the manufacture of antitoxins and other biological products. This serum, obtained under aseptic precautions, has obviously an advantage, and has in our hands proven entirely satisfactory for routine work.

In either case the serum is to be decanted into clean, sterile test-tubes provided with cotton plugs, after which it must be immediately sterilized. For this purpose the

method suggested by Councilman and Mallory is now generally used. It is as follows: Place the test-tubes containing the serum in a slanting position in a *dry air* sterilizer and heat them to from 80° 90° C. for a time necessary to solidify the serum. After this they are kept for twenty minutes on three successive days in the steam sterilizer at 100° C. They should be kept at room temperature between

FIG 19

Chamber for sterilizing and solidifying blood-serum. (Koch.)

the exposures to the steam. After this treatment the serum should be sterile.

Serum thus prepared may be kept from drying by burning off in the gas flame the excess of cotton protruding from the ends of the tubes and then forcing down upon the cotton plugs clean, new, corks that have been sterilized by steam under pressure. (Ghriskey.)

To secure satisfactory results by this method several

precautions should be noted, viz.: The solidification of the serum in the dry air sterilizers must be complete, else its surface will be rough and broken by bubbles; the same results if the temperature in the dry air sterilizer is brought up too rapidly.

Serum prepared in this way is neither clear nor colorless. This is ordinarily not a disadvantage. The popularity of the method is due to its simplicity, the rapidity with which a satisfactory serum may be prepared and especially to the fact that the rigid precautions against contamination observed in the older methods, where sterilization at low temperature was practised, are not essential to success, since even though such contaminations occur they are eliminated by the high temperatures used in this procedure.

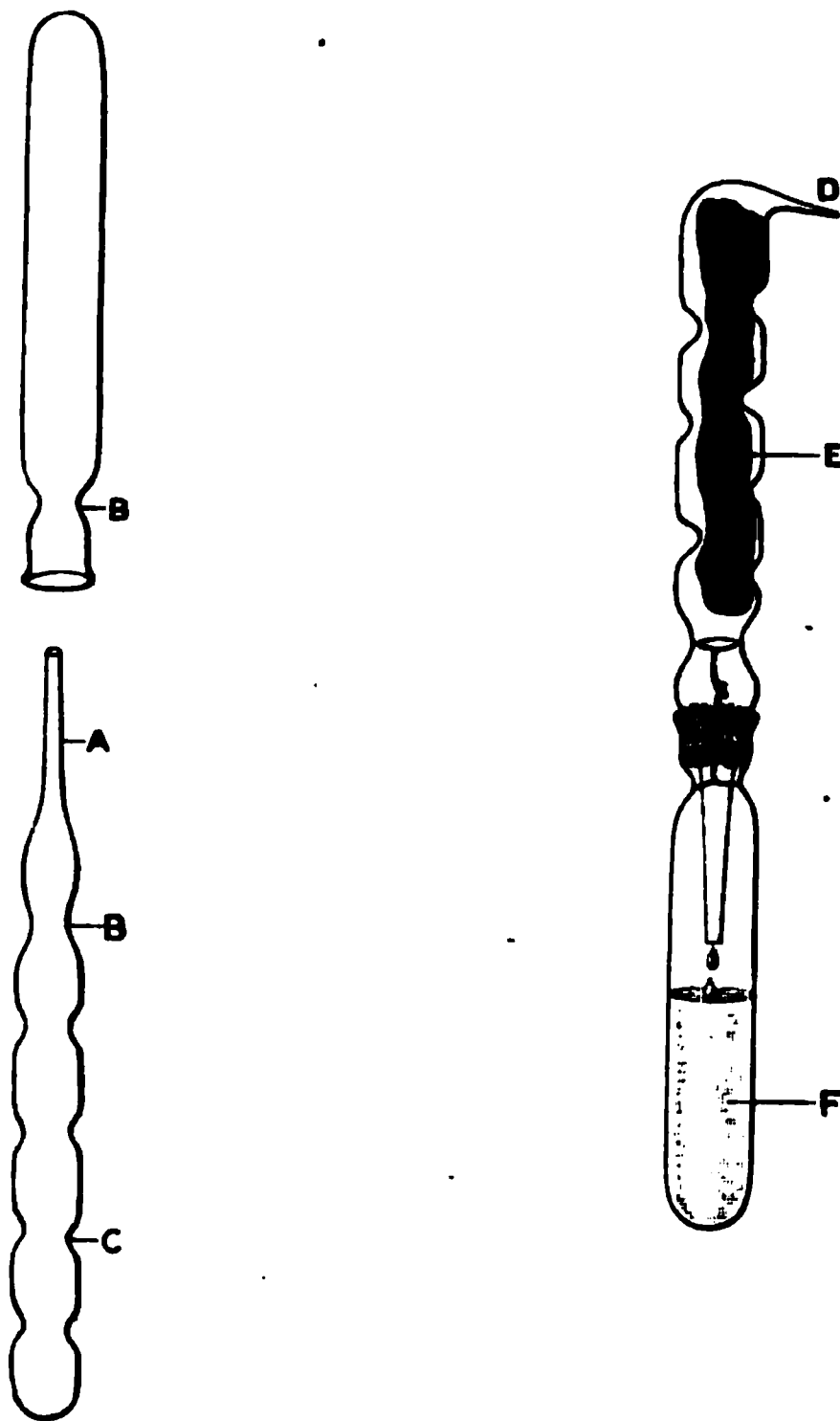
Blood-serum from Small Animals. — For special purposes it is often desirable to secure blood serum under strictly aseptic precautions from particular species of animals, many of them being small. To this end there have been devised a number of handy methods. That which in our hands has proven the simplest and generally most useful is the Rivas modification¹ of Latapie's method. It is as follows:

The Rivas apparatus is constructed from two test-tubes about 15 x 180 mm. in size. The mouth of one test-tube is drawn out into a long narrow neck 1 cm. in diameter and about 5 cm. in length. Three or four points on the side of the tube are softened in the flame of a blowpipe, and the softened glass driven inward by means of a piece of pointed wood. This gives supports on the interior of the tube to hold the coagulated blood in place. Between the long narrow neck and the body of the tube a constriction is

¹ University of Pennsylvania Medical Bulletin, 1904, vol. xvii, p. 295.

formed by drawing out the tube while heated. The second tube also has a similar constriction about 20 cm. from its mouth.

FIG. 20



Rivas apparatus for collecting blood-serum: *A*, long narrow neck on first tube; *B*, constriction on tubes near mouth; *C*, invaginations on first tube; *D*, small cannula drawn out on extremity of first tube; *E*, blood-clot, and *F*, blood-serum collected in bottom of second tube.

The two tubes are now fitted together by inserting the one with the long narrow neck into the second tube; a small

amount of cotton being first carefully folded around the neck of the first tube, so as to prevent the entrance of dust. The two tubes are then fastened together by means of a wire twisted around the constriction at the neck of each tube, and the apparatus is then wrapped in cotton and sterilized in a hot-air sterilizer.

Before using the apparatus the extremity of the first tube is heated in the gas-flame, and by touching this point with a piece of pointed glass rod it is gently drawn out into a fine cannula. When the animal has been prepared for the operation and a vessel exposed, the point of the cannula is snipped off with a sterile scissors, when the point of the cannula is inserted into the vessel. The pressure of blood is sufficient to fill the first tube. The point of the cannula is now removed from the vessel and sealed in a gas-flame. The apparatus is laid aside in an almost horizontal position until the blood has become completely coagulated. It is then inverted and set aside for the serum to separate and trickle down through the narrow neck of the first tube and collect in the second tube. When this has occurred, the wire holding the two tubes together is unwound, and the first tube is removed and the second plugged with a well-fitting sterile cotton plug, when the serum may be preserved in the tube for several days without danger of contamination.

Preservation of Blood-serum.—It is sometimes desirable to preserve blood-serum in a fluid state. This can be done by the fractional method of sterilization at low temperatures, already described, or with much less effort, and without the use of heat, by a method that we have found very satisfactory. In the course of Kirschner's investigations chloroform was shown to possess decided disinfectant properties; as it is quite volatile, it is easily got rid of when

its disinfectant or antiseptic properties are no longer required. If, therefore, the serum to be preserved be placed in a closely stoppered flask and enough chloroform added to form a thin layer, about 2 mm., on the bottom, the serum may be kept indefinitely without contamination, so long as the chloroform is not permitted to evaporate. • This latter provision is one on which success depends. If the vessel containing the mixture of chloroform and serum be not *tightly* corked, the chloroform vapor escapes pretty rapidly and exerts no preservative action. In fact, bacteria will grow uninterruptedly in a cotton-stoppered test-tube containing bouillon to which chloroform has been added. When required for use, the serum is decanted into test-tubes, which are then placed in a water-bath at about 50° C. until all the chloroform has been driven off; this can be determined by the absence of its characteristic odor. The serum may then be solidified, sterilized by heat, and employed for culture purposes. We have found serum so preserved to answer all requirements as a culture-medium.

MILK.

Fresh milk should be allowed to stand over night in an ice-chest, the cream then removed, and the remainder of the milk pipetted into test-tubes, about 8 c.c. to each tube, and sterilized by the intermittent process, at the temperature of steam, for three successive days.

The separation of the cream may be accelerated and rendered more complete if the cylinder containing the milk be placed in the steam sterilizer for fifteen minutes before it is placed in the ice-chest.

The cream is best separated from the milk by the use of

a cylindrical vessel with a stopcock at the bottom, by means of which the milk, devoid of cream, may be drawn off. A Chevalier creamometer with a stopcock at the bottom serves the purpose very well. It should be covered while standing.¹

Milk may be used as a culture-medium without any addition to it, or, before sterilizing, a few drops of litmus tincture may be added, just enough to give it a pale-blue color. By this means it will be seen that different organisms bring about different reactions in the medium: some producing alkalies, which cause the blue color to be intensified; others producing acids, which change it to red; while others bring about neither of these changes. Similarly litmus solution is often added to gelatin and agar-agar for the same purpose.

Milk may also be employed as a solid culture-medium by the addition to it of gelatin or agar-agar in the proportions given for the preparation of ordinary nutrient gelatin or agar-agar. It has, however, in this form the disadvantage of not being transparent, and can therefore best be used for the study of those organisms which grow upon the surface of the medium without causing liquefaction.

Nutrient gelatin and agar-agar can also be prepared from neutral milk-whey, obtained from milk after precipitation of the casein.

Litmus-whey Milk.—An important differential medium is milk-whey to which litmus tincture has been added. A number of methods for its preparation are in use, but the one employed by Durham seems to be the most satisfactory.

¹ For some time past we have been using what is technically known as "separator milk"—i. e., the fluid left after milk has been deprived of its fat (cream) by centrifugal force.

Briefly it is as follows: fresh milk, free from antiseptic adulterations, is gently warmed and clotted with essence of rennet. The whey is strained off and the clot hung up to drain in a piece of muslin. The whey, which is somewhat turbid and yellow, is then cautiously neutralized with a 4 per cent. citric acid solution, neutral litmus solution being used as the indicator. It is then heated upon a water-bath to 100° C. for about half an hour; thereby nearly the whole of the proteid is coagulated. It is then filtered clear and neutral litmus solution is added until it is of a distinct purple color. If the filtered whey is cloudy, let it stand in a cold place for a day or two and decant off the clear supernatant portion or pass it through a Berkefeld filter. The whey should never be heated above 100° C. or neutralized with mineral acids, otherwise there is a danger of so modifying the milk-sugar present as seriously to impair the usefulness of the medium. When properly prepared, the medium is free from proteid, and contains only water, lactose, the salts of the milk, and a small quantity of a body suggestive of dextrose or galactose. The medium is of great utility in detecting the power of bacteria to cause acid fermentation in a non-proteid medium containing a fermentable sugar; and for observing the variations of this power in closely allied though not identical species.

DUNHAM'S PEPTONE SOLUTION.

The medium known as Dunham's solution is prepared according to the following formula:

Dried peptone	1.0 part
Sodium chloride	0.5 part
Distilled water	100.0 parts

It is usually of a neutral or slightly alkaline reaction and neutralization is not, therefore, necessary. It is filtered, decanted into tubes or flasks, and sterilized in the steam sterilizer in the ordinary way. The most common use to which this solution is put is in determining if the organism under consideration possesses the property of producing indol as one of its metabolic products. It is essential for accuracy that the preparation of dried peptone employed should be as nearly chemically pure as is possible, and indeed the other ingredients should be correspondingly free from impurities. Gorini¹ calls attention to the fact that impurities in the peptone, particularly the presence of carbohydrates, so interfere with the production of indol by certain bacteria that otherwise produce it, that it is oftentimes impossible, under such circumstances, to obtain the characteristic color-reaction of this body, and where it is obtained it is always after a much longer time than is the case where peptone free from these substances has been used.

Peckham has also demonstrated that where bacteria have the property of forming indol and also of fermenting carbohydrates, their proteolytic function, as evidenced by the appearance of indol as a product of metabolism, may be completely suppressed by the addition of such fermentable carbohydrates as glucose, saccharose, and lactose to the proteid solution in which they are developing.²

Gorini suggests the advisability of testing the purity of all peptone preparations before using them, by means of the reaction that they exhibit with Fehling's alkaline copper solution. Under the influence of this reagent pure peptone in solution gives a violet color (the biuret reaction), which

¹ *Centralblatt für Bakteriologie und Parasitenkunde*, 1893, vol. xiii, p. 790.

² See *Journal of Experimental Medicine*, 1897, vol. ii, p. 559.

remains permanent even after boiling for five minutes. If, instead of a violet color, there appears a red or reddish-yellow precipitate, the peptone should be discarded, as in his experience no indol is produced from peptone giving this reaction. Both the peptone solution and that of the copper (particularly the latter) should be relatively dilute in order for the reaction to be successful.

LACTOSE LITMUS-AGAR, OR LITMUS-GELATIN OF WURTZ.

A medium of much use in the differentiation of bacteria is that recommended by Wurtz, consisting of slightly alkaline nutrient agar-agar, to which from 2 to 3 per cent. of lactose and sufficient litmus tincture to give it a pale-blue color have been added. Bacteria capable of causing fermentation of lactose when grown on this medium develop into colonies of a pale-pink color and cause, likewise, a reddening of the surrounding medium, owing to the production of acid as a result of their action upon the lactose; while other bacteria, incapable of such fermentative activities, grow as pale-blue colonies and cause no reddening of the surrounding medium. It is especially useful in the differentiation of the bacillus of typhoid fever, which does not possess the property of bringing about fermentation of lactose, from other organisms that simulate it in many other respects, but which do possess this property.

Its preparation is as follows: to nutrient agar-agar or gelatin, the alkalinity of which is such that 1 c.c. will require 0.1 c.c. of a 1 : 20 normal sulphuric-acid solution to neutralize it, lactose is added in the proportion of 2 or 3 per cent.; it is then decanted into test-tubes and sterilized in

the usual way. When sterilization is complete enough *sterilized* litmus tincture should be added to each tube to give a decided, though not very intense, blue color. This must be done carefully, to avoid contamination of the tubes during manipulation. It is better not to add the litmus tincture before sterilizing the tubes, as its color-characteristics are altered by contact with organic matters under the influence of heat. This medium is used for both test-tube and plate cultivation, just as is ordinary agar-agar and gelatin.

LÖFFLER'S BLOOD-SERUM MIXTURE.

Löffler's blood-serum mixture consists of one part of neutral meat-infusion bouillon, containing 1 per cent. of grape-sugar, and three parts of blood-serum. This mixture is placed in test-tubes, sterilized, and solidified in exactly the way given for blood-serum. It requires for its solidification a somewhat higher temperature and a longer exposure to this temperature than does blood-serum to which no bouillon has been added. (See also the Councilman-Mallory method.)

THE SERUM-WATER MEDIUM OF HISS.

A medium which has been found very serviceable in the differentiation between closely related bacteria is prepared by mixing one part of blood-serum (either horse or bovine) and three parts of distilled water. This is neutralized, and heated in a water-bath or an Arnold steam sterilizer until it becomes opalescent. A 5 per cent. aqueous solution of litmus is then added in the proportion of 1 per cent. Any one of the carbohydrates, as dextrose, lactose, saccharose,

levulose, mannite, etc., is then added in the proportion of 1 per cent. The finished medium is then placed in test-tubes. The medium must be sterilized in an Arnold steam sterilizer, and it is advisable to allow the sterilizer to remain uncovered during the process of sterilization to avoid excessive heating of the medium.

The relative degree of acidity produced, with or without coagulation, with or without gas-production, and with or without reduction of the litmus, in a series of tubes of this medium containing the different carbohydrates serves to differentiate between related species of bacteria. For instance, the colon bacillus produces an acid reaction with coagulation and gas-formation with some of the carbohydrates, while the typhoid bacillus produces a lower degree of acidity with coagulation, but without gas-production. Similarly, the different types of the dysentery bacillus may be differentiated by means of their effects on the different carbohydrates in this medium.

GUARNIARI'S GELATIN-AGAR MIXTURE.

For special work, particularly with the organism of pneumonia (*bacterium pneumoniæ*) the gelatin-agar mixture recommended by Guarniari is of very great service. It should be exactly neutral in reaction, and should possess the following ingredients:

Meat infusion	950 c.c.
Sodium chloride	5 grams
Peptone	25 to 30 grams
Gelatin	40 to 60 grams
Agar-agar	3 to 4 grams
Water	50 c.c.

The agar-agar should be completely dissolved separately in about 100 c.c. of water in the autoclave while the other ingredients are being prepared. The latter should be filtered and the dissolved agar-agar added to the filtrate.

A complete list of the special media would be too voluminous for a book of this size. For their description the reader is referred to the current literature. Those that have been given above will suffice for obtaining a clear understanding of the principles of the subject. In the chapters upon the Pathogenic Bacteria such special media as have proved of use for purposes of identification and differentiation are described in detail.

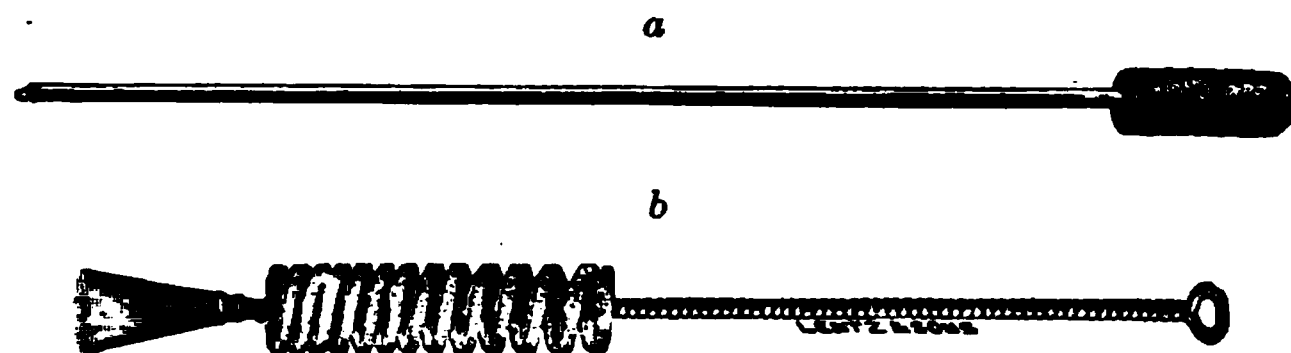
CHAPTER VI.

Preparation of the Tubes, Flasks, etc., in which the Media are to be Preserved.

WHILE the media are in course of preparation it is well to get the test-tubes and flasks ready for their reception, and it is essential that they should be as clean as it is possible to make them. For this purpose it is advisable that both new tubes and those which have previously been used should be boiled for about thirty to forty-five minutes in a 2 to 3 per cent. solution of common soda; it is not necessary to be exact as to strength, but it should not be weaker than this. At the end of this time they are to be carefully swabbed out with a cylindrical bristle brush, preferably one with a reed handle (Fig. 21, *a*), as those with wire handles are apt to break through the bottoms of the tubes, though Messrs. Lentz & Sons, of Philadelphia, have in large part eliminated this objection from the wire-handle brush depicted in Fig. 21, *b*. All traces of adherent material should be carefully removed. When the tubes are quite clean they may be rinsed in a warm solution of commercial hydrochloric acid of the strength of about 1 per cent. This is to remove the alkali. They are then to be thoroughly rinsed in clear, running water, and stood top down until the water has drained from them. When dry they are to be plugged with raw cotton; this requires a little practice before it can be properly done. The cotton should be introduced into the mouths of the tubes in such a way that no cracks or creases

exist. The plug should fit neither too tightly nor too loosely, but should be just firmly enough in position to sustain the weight of the tube into which it is placed when held up by the portion which projects from and overhangs the mouth of the tube. The tubes thus plugged are now to be placed upright in a wire basket and heated for one hour in the hot-air sterilizer at a temperature of about 150°C . A very good guide for this process of sterilization is to observe the tubes from time to time, and as soon as the cotton has become a very light-brown color, not deeper than a dark-cream tint, to consider sterilization complete. The tubes are then removed and allowed to cool.

FIG. 21



Brushes for cleaning test-tubes.

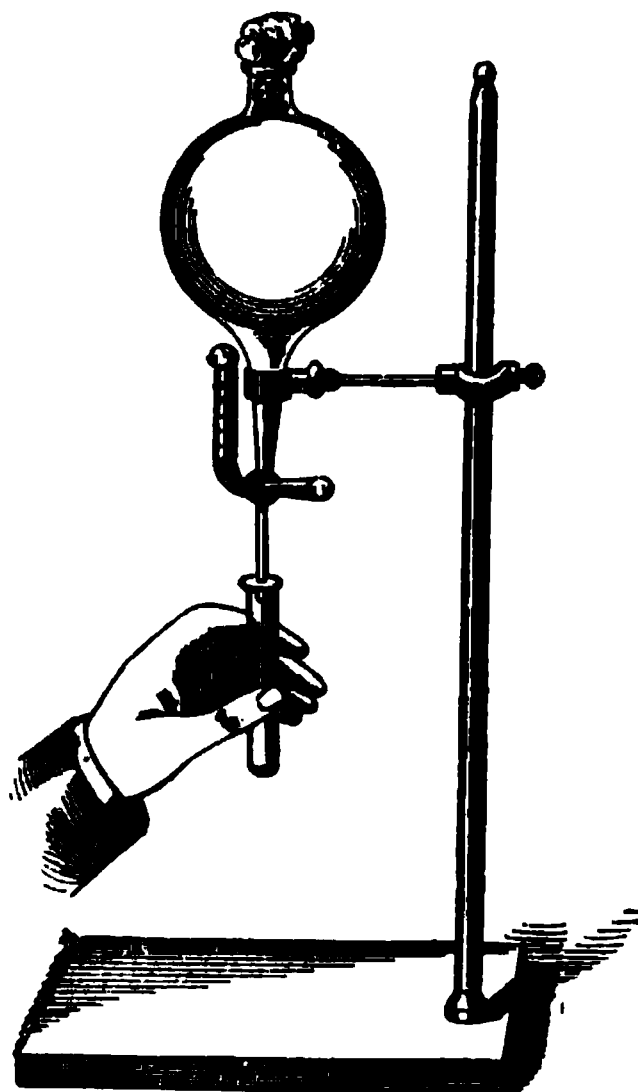
The cotton used for this purpose should be the ordinary cotton batting of the shops, and not absorbent cotton; the latter becomes too tightly packed, and is, moreover, much too expensive for this purpose.

Care should be taken not to burn the cotton, otherwise the tubes will become coated with a dark-colored, empyreumatic, oily deposit, which necessitates recleansing.

Filling the Tubes.—When the tubes are cold they may be filled. This is best accomplished by the use of a separating funnel, such as is shown in Fig. 22. The liquefied medium is poured into this funnel, which has been carefully washed,

and by pressing the pinchcock with which the funnel is provided the desired amount of material (5-10 c.c.) may be allowed to flow into the tubes held under its opening. It is not necessary to sterilize the funnel, for the medium is to be subjected to this process as soon as it is in the test-tubes.

FIG. 22



Funnel for filling tubes with culture-media.

Care should be taken that none of the medium is dropped upon the mouth of the test-tube, otherwise the cotton plug becomes adherent to it, and is not only difficult to remove, but presents a very untidy appearance and interferes materially with the manipulations.

As soon as the tubes have been filled they are to be sterilized either in the steam sterilizer at 100° C. for fifteen

minutes on each of three successive days, being kept during the intervals at room temperature, or they may be sterilized by a single exposure of 15 minutes in the autoclave to a temperature equivalent to steam under about one atmosphere of pressure.

When sterilization is complete and the medium in the tubes is still liquid, some of them may be placed in a slanting position, at an angle of about ten degrees with the surface on which they rest, and the medium allowed to solidify in this position. These are for the so-called slant-cultures. The remainder may solidify in the erect position; these serve for making plates.

CHAPTER VII.

Technique of Isolating Bacteria in Pure Culture by the Plate and the Tube Method.

PLATES.

THE plate method can be employed with both agar-agar and gelatin. It cannot be practised with blood-serum, because the serum when once solidified cannot again be liquefied.

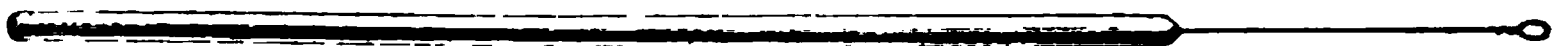
Plates are usually referred to as "a set." This term implies three individual plates, each representing a mixture of organisms in a higher state of dilution. The first plate is known usually as "the original," or "plate No. 1," the first dilution from this as "plate No. 2," and the second as "plate No. 3."

In the preparation of a set of plates the following are the steps to be observed:

Three tubes, each containing from 7 to 9 c.c. of gelatin or agar-agar, are placed in a warm water-bath until the medium has become liquid. If agar-agar is employed, this is accomplished at the boiling-point of water; if gelatin is used, a much lower temperature suffices (35°–40° C.). When liquefaction is complete the temperature of the water, in the case of agar-agar, must be reduced to 41°–42° C., at which temperature the agar-agar remains liquid, and the organisms may be introduced into it without fear of destroying their vitality. The medium being now liquid and

of the proper temperature, a very small portion of the mixture of organisms to be studied is taken up with a sterilized platinum wire (Fig. 23, *a*) about 5 cm. long, twisted into a small loop at one end and fused into a bit of glass rod, which serves as a handle, at the other extremity. This loop is one of the most useful of bacteriological instruments, as there is hardly a manipulation into which it does not enter. Under no circumstances is it to be employed without having been passed through a gas-flame until quite hot, for the purpose of sterilization. One should form a habit of never taking up one of these platinum-wire needles, as

FIG. 23

a*b*

Looped and straight platinum wires in glass handles.

they are called, for they are curved and straight (Fig. 23, *b*) as well as looped, without passing it through a flame; and the sooner the beginner learns to do this as a reflex action, the sooner does he eliminate one of the possible sources of error in his work. It must be remembered, though, that it should not be used when hot, otherwise the organisms taken upon it will be killed by the high temperature; after sterilization in the flame one waits for a few seconds until it is cool before using.

A minute portion of the material under consideration is transferred with the sterilized loop into tube No. 1, "the original," where it is thoroughly disintegrated by gently

rubbing it against the sides of the tube. The more carefully this is done the more uniform will be the distribution of the organisms and the better the results. The loop is then again sterilized and three of its loopfuls are passed, *without touching the sides of the tube*, from "the original" into tube No. 2, where they are carefully mixed. Again the loop is sterilized, and again three dips are made from tube No. 2 into tube No. 3. This completes the dilution. The loop is now sterilized before laying it aside.

FIG. 24

Levelling tripod with glass cooling chamber for plates.

During this manipulation, which must be done quickly if agar-agar be employed, the temperature of the water in the bath in which the tubes stand should never be lower than 39° C., and never higher than 43° C. If it falls below 38° C., the agar-agar solidifies, and can only be redissolved at a temperature that would be destructive to the organisms which may have been introduced into the tubes. This is

not of so much moment with gelatin, since it may readily be redissolved at a temperature not detrimental to the organisms with which the tubes may have been inoculated. When completed the dilutions are poured into sterilized Petri dishes to cool and solidify, thereby fixing the bacteria so that the individuals may develop into their characteristic colonies and be so separated from one another as to permit of easy isolation in pure culture.

The Petri dish (Fig. 25) is of glass; round in form, about 8 or 9 cm. in diameter and 1.5 to 2 cm. deep, with a loosely fitting cover. To hasten the solidification of the medium

FIG. 25



Petri double dish, now generally used instead of plates.

the dishes may be cooled by placing them upon a cold surface, such as is provided by the glass cooling stage (Fig. 24), when packed with ice, or on the metal cooler, shown in Fig. 26, through which cold water circulates. The plates are labeled to correspond with their respective dilutions and are then set aside, protected from dust and light until colony development begins. In the case of gelatin the plates must not be maintained at a temperature higher than that of an ordinary living room, about 20° to 22° C. being the most favorable. In the case of agar-agar the plates may be maintained at the temperature of the animal body, *i. e.*, between 37° and 38° C.

TUBES.

Esmarch Tubes.—A useful modification of the plating method just described is that suggested by von Esmarch. It insures the greatest security from contamination by extraneous organisms and requires the least amount of apparatus. It differs from the other methods thus: the dilutions having been prepared in tubes contain a smaller amount of medium than usual—as a rule, not more than 5 to 6 c.c.—are, instead of being poured upon plates or into dishes, spread over the inner surface of the tubes containing

FIG. 26

Metal cooling stage.

them, and, without removing the cotton plugs, solidified in this position. The tubes then present a thin cylindrical lining of gelatin or agar-agar, upon which the colonies develop. In all other respects the conditions for the growth of the organisms are the same as in flat plates.

The solidification of the media on the inner sides of the tubes is best accomplished by rolling them upon a block of ice (Fig. 27), after the plan devised by Booker in 1887 in the Pathological Laboratory of the Johns Hopkins University. In this method a small block of ice only is needed.

It is levelled and held in position by being placed upon a towel in a dish. A horizontal groove is melted in the upper surface of the ice with a test-tube of hot water. The tubes to be rolled are then held in an almost—*not quite*—horizontal position and twisted between the fingers until the sides are moistened by the contents to within about 1 cm. of the cotton plug, care being taken that the gelatin *does not touch* the cotton, otherwise the latter becomes adherent to the sides of the tube and is difficult to remove. The tube

FIG. 27

Demonstrating Booker's method of rolling Esmarch tubes on a block of ice.

is then placed in the groove in the ice and rolled until its contents are solid.

There is an erroneous impression that Esmarch tubes are not a success when made from ordinary nutrient agar-agar because of the tendency of this medium to shrink and slip to the bottom of the tube. This slipping down of the agar-agar is due to the water, which is squeezed from it during solidification, getting between the medium and the walls of the tube. This can easily be overcome by allowing the

rolled tubes to remain in a nearly horizontal position for twenty-four hours after rolling them, the mouth of the tube being about 1 cm. higher than the bottom. During this time the margin of the agar-agar nearest the cotton plug dries and becomes adherent to the walls of the tube, while the water collects at the most dependent point—*i. e.*, the bottom of the tubes. After this they may be retained in the upright position without danger of the agar-agar slipping down.

In both the plates and tubes, if the dilutions of the number of organisms have been properly conducted, the results will be the same. The original plate or tube, as a rule, will be of no use because of the great number of colonies in it; plate or tube No. 2 may be of service; but plate or tube No. 3 will usually contain the organisms in such small numbers that there will be nothing to prevent the characteristic development of the colonies originating from them.

For reasons of economy the “original,” tube No. 1, is sometimes substituted by a tube containing normal salt-solution (0.6 to 0.7 per cent. of sodium chloride in water), which is thrown aside as soon as the dilutions are completed, and only plates or tubes Nos. 2 and 3 are made.

The Serial Tube Method of Separation.—Another method for the separation of bacteria and their isolation as single colonies consists in the making of dilutions upon the surface of solid media, such as potato, coagulated blood-serum, agar-agar, and gelatin. In pursuance of this method one selects a number of tubes containing the medium set in a slanting position. With a platinum needle a bit of the substance to be studied is smeared upon tube No. 1; without sterilizing the needle it is passed in succession over the surface of the medium in tubes Nos. 2, 3, 4, etc. When develop-

ment has occurred essentially the same conditions as regards separation of the colonies will be found as when plates are poured. If a slanted medium be employed, about the most dependent angle of which water of condensation has accumulated, as blood-serum, agar-agar, and potato, the dilutions may be made in this fluid, and this is then to be carefully smeared over the solid surface of the medium. The tubes thus treated should be kept in an upright position to prevent the fluid flowing over the surface. When sufficiently developed, single colonies may be isolated with comparative ease from tubes prepared in this manner. (See also method for the isolation of *bacillus diphtheriæ* on blood-serum.)

CHAPTER VIII.

The Incubating Oven—The Safety Burner Employed in Heating the Incubator—Thermo-regulator—Gas-pressure Regulator.

THE INCUBATOR.

WHEN the plates have been made it must be borne in mind that for the development of certain forms of bacteria a higher temperature is necessary than for the growth of others. The pathogenic or disease-producing organisms grow more luxuriantly at the temperature of the human body (37.5° C.) than at lower temperatures; whereas for the ordinary saprophytic forms almost any temperature between 18° and 37° C. is suitable. It therefore becomes necessary to provide a place in which a constant temperature favorable to the growth of the pathogenic organisms can be maintained. For this purpose a number of different forms of apparatus have been devised. They are all based upon the same principles, however, and a general description of the essential points involved in their construction will be all that is needed here.

The apparatus known as the incubator, or brooding-oven, is a copper chamber (Fig. 28) with double walls, the space between which is filled with water. The incubating-chamber has a closely-fitting double door, inside of which is a door of glass through which the contents of the chamber may be inspected without actually opening it. The whole apparatus is encased in either asbestos-boards or thick felt, to prevent radiation of heat and consequent fluctuations in tempera-

ture. In the top of the chamber is a small opening through which a thermometer projects into its interior. At either corner, leading into the space containing the water, are

FIG. 28

Incubator used in bacteriological work.

other openings for the reception of another thermometer and a thermo-regulator, and for refilling the apparatus as the water evaporates. On the side is a water-gauge for showing the level of the water between the walls. The

object of the water-chamber, which is formed by the double-wall arrangement, is to insure, by means of the warmed water, an equable temperature in all parts of the apparatus—at the top as well as at the sides, back, and bottom; the apparatus should be kept filled with water, otherwise the purpose for which it is constructed will not be served. When the chamber between the walls is filled with water heat is supplied by a gas-flame placed beneath it.

FIG. 29

Koch's safety burner.

The burner employed in heating the incubator was originally devised by Koch, and is known as "Koch's safety burner" (Fig. 29¹). It is a Bunsen burner provided with an arrangement for automatically turning off the gas-supply

¹ There are now many modifications of the original form.

and thus preventing accidents should the flame become extinguished at a time when no one is near. The gas-cock by which the gas is turned on and off is provided with a long arm which is weighted, and which, when the gas is turned on and *burning*, rests upon an arm attached to the side of a revolving, horizontal disk that is connected with the free ends of two metal spirals which are fixed by their other ends in opposite directions on either side of the flame and heated by it. If by draughts or any other accident the flame becomes extinguished, the metal spirals cool, and in cooling contract, twist the horizontal disk in the opposite direction, and by thus removing the support allow the weighted arm of the gas-cock to fall. By its falling the gas-supply is turned off.

Thermo-regulators.—The regulation and maintenance of the proper temperature within the incubator are accomplished by the employment of an automatic thermo-regulator or thermostat.

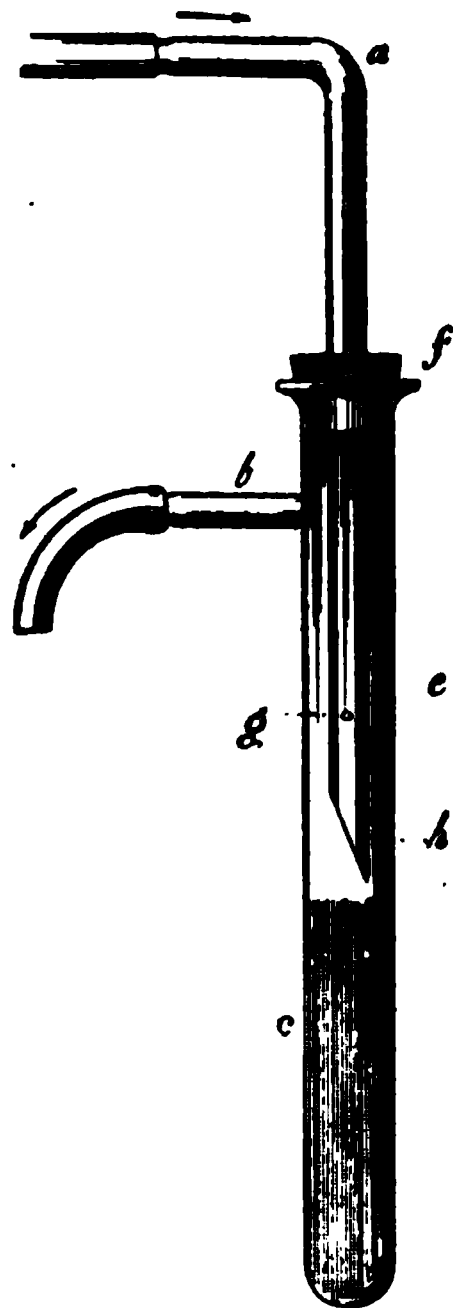
The common form of thermo-regulator used for this purpose is constructed upon principles involving the expansion and contraction of fluids under the influence of heat and cold. By means of this expansion and contraction the amount of gas passing from the source of supply to the burner may be either diminished or increased as the temperature of the substance in which the regulator is placed either rises or falls.

The simplest form of thermo-regulator which serves to illustrate the principles is seen in Fig. 30. It consists of a glass cylinder, *e*, having a communicating branch tube *b*, and rubber stopper *f*, through which projects the bent tube *a*. The tube *a* is ground to a slanting point at the extremity which projects into the tube *e*, and is provided a short dis-

tance above this point with a capillary opening, *g*, in one of its sides.

When ready for use the cylinder *e* is filled with mercury up to about the level shown in the figure. It is then allowed to stand, or is suspended, in the bath the temperature of which it is to regulate. The rubber tubing coming from the gas-supply is attached to the outer end of the glass tube *a*, and the tube going to the burner is slipped over the branch tube *b*. The gas is turned on and the burner lighted and placed under the bath. The gas now streams through the tube *a* into the cylinder *e* and out at *b* to the burner; but as the temperature of the bath rises the mercury contained in the cylinder *e*, under the influence of the elevated temperature, begins to expand, and, as a continuous rise in temperature proceeds, the expansion of the mercury accompanies it and gradually closes the slanting opening *h* of tube *a*. In this way the supply of gas becomes diminished and the rise in temperature of the bath will be less rapid, until finally the opening at *h* will be closed entirely, when the supply of gas to the burner will now be limited to that passing through the capillary opening *g*. This is not sufficient to maintain the highest temperature reached,

FIG. 30



Mercurial thermo-regulator.

and as cooling begins a gradual contraction of the mercury occurs until there is again an outflow of gas from the opening *h*, when the temperature again rises. This contraction and expansion of the mercury in the regulator continues until eventually a point is reached at which its position in the cylinder *e* allows of the passage of just enough gas from the opening *h* to maintain a constant temperature and, therefore, a constant degree of expansion of the mercury in the tube *e*. This, in short, is the principle on which thermo-regulators are constructed; but it must be borne in mind that a great deal of detail exists in the construction of an accurate instrument. The number of different forms of this apparatus is comparatively large, and each form has its special merits.

The value—that is, the delicacy—of the thermo-regulator depends upon a number of factors, all of which it would be useless to describe in a book of this kind; but in general it may be said that the essential points to be observed in selecting a thermo-regulator depend in the main upon the temperatures at which it is to be used. For low temperatures, regulators containing such fluids as ether, alcohol, and calcium chloride solution, which expand and contract rapidly and regularly under slight variations in temperature, are commonly employed; whereas for temperatures approaching the boiling-point of water mercury is most frequently used.

The temperature of the incubator is to be regulated, then, by the use of some such form of apparatus as that just described. It should be of sufficient delicacy to prevent a fluctuation of more than 0.2° C. in the temperature of the air within the chamber of the apparatus.

Gas-pressure Regulators.—A gas-pressure regulator is not rarely intervened between the gas-supply and the thermo-

regulator. This apparatus has for its object the maintenance of a constant pressure of the gas going to the thermo-regulator. There are several forms of regulator in use, but they do not accomplish the object for which they are designed.

The instrument most commonly employed, the apparatus of Moitessier (Fig. 31), is based on somewhat the same principles as the large regulators seen at the manufactories of illuminating-gas. Such apparatus act very well when

FIG. 31

Moitessier's gas-pressure regulator.

employed on the large scale, as one sees them at the gas-works; but when applied to the limited and sudden fluctuations seen in the gas coming from an ordinary gas-cock are practically useless. They are too gross in their construction, and act only under comparatively great and gradual fluctuations in pressure. If a good form of thermo-regulator be employed, there is no necessity for the use of any of the pressure-regulators thus far introduced.

CHAPTER IX.

The Study of Colonies—Their Naked-eye Peculiarities and Their Appearance Under Different Conditions—Differences in the Structure of Colonies from Different Species of Bacteria—Stab-cultures—Slant-cultures.

THE plates of agar-agar which have been prepared from a mixture of organisms and have been placed in the incubator, and those of gelatin which have been maintained at the ordinary temperature of the room, are usually ready for examination after from twenty-four to forty-eight hours. They will be found marked here and there by small points or little islands of more or less opaque appearance. In some instances these will be so transparent that it is with difficulty one can see them with the naked eye. Again, they may be of a dense, opaque appearance; at one time sharply circumscribed and round, again irregular in their outline; here a point will present one color, there perhaps another. On gelatin some of the points will be seen to be lying on the surface of the medium, others will have sunk into little depressions, while at still other points the clear gelatin will be marked by more or less saucer-shaped pits containing opaque fluid.

Place the plate containing these points upon the stage of a microscope and examine them with a low-power objective, and again differences will be observed. Some of these minute points will be finely granular, others coarsely so; some will present a radiated appearance, while a neighbor may be concentrically arranged; here nothing particularly characteristic will present, there the point may resolve itself into a mass having somewhat the appearance of a

little pellicle of raw cotton. All these differences, and many more, aid us in saying that these objects must be different in their constitution. With a pointed platinum needle take up a bit of one of these small islands, prepare it for microscopic examination (see chapter on Stained Cover-slip Preparations), and examine it under the high-power oil-immersion objective, with access of the greatest amount of light afforded by the illuminator of the microscope. The preparation will be seen to be made up entirely of bodies of the same shape; they will all be spheres, or ovals, or rods, but not a mixture of these forms, if proper care in the manipulation had been taken. Examine in the same way a neighboring spot which possesses different naked-eye appearances, and often it will be found to consist of bodies of an entirely different appearance from those seen in the first preparation.

These spots or islands on the surface of the plates are colonies of bacteria, differing severally, not only in their gross appearances, the one from the other, but, as our cover-slip preparations show, in the morphological characteristics of the individual organisms composing them.

If from one of these colonies a second set of plates be prepared, the peculiarities which were first observed in it will be reproduced in all of the new colonies which develop; each will be found to consist of the same organisms as the colony from which the plates were made. In other words, these peculiarities are constant under uniform conditions.

The appearance of the colonies developing from all organisms is regulated by their location in the medium in which they are growing. When deep down in the medium they are usually round, oval, or lozenge-shaped; whereas when on the surface of the gelatin or agar they may take quite a

different form. This is purely a mechanical effect due to the pressure of, or resistance offered by, the medium surrounding them, and is always to be borne in mind, otherwise false interpretations may be made.

Pure Cultures.—If from one of these small colonies a bit be taken upon the point of a *sterilized* platinum needle and introduced into a tube of sterilized gelatin or agar-agar, the growth that results will be what is known as a “pure culture,” the condition to which all organisms must be brought before a systematic study of their many peculiarities is begun. Sometimes several series of plates are necessary before the organisms can be obtained pure, but by patiently following this plan the results will ultimately be satisfactory.

Test-tube Cultures; Stab-cultures; Smear-cultures.—After separating the organisms the one from the other by the plate method just described, they must be isolated from the plates as pure stab- or smear-cultures.

This is done in the following way: decide upon the colony from which the pure culture is to be made. Select preferably a small colony and one as widely separated from other colonies as possible. Sterilize in a gas-flame a straight platinum-wire needle. The glass handle of the needle should be drawn through the flame as well as the needle itself, otherwise contamination from this source may occur. When it is cool, which is in five or ten seconds, take up carefully a portion of the colony. Guard against touching *anything but the colony*. If during manipulation the needle touches *anything else whatever* than the colony from which the culture is to be made, it must be sterilized again. This holds not only for the time before touching the colony, but also during its passage into the test-tube from the colony; otherwise there is no guarantee that the growth resulting

from the inoculation of this bit of colony into a fresh sterile medium will be pure.

In the meantime have in the other hand a test-tube of sterile medium: gelatin, agar-agar, or potato. This tube is held across the palm of the hand in an almost horizontal position with its mouth pointing out between the thumb and index-finger and its contents toward the body of the worker. With the disengaged fingers of the other hand holding the needle the cotton plug is removed from the tube by a twisting motion and placed between the index and second fingers of the hand holding the tube, in such a way that the portion of the plug which fits into the mouth of the test-tube looks toward the dorsal surface of the hand and *does not touch any portion of the hand*; this is accomplished by placing *only the overhanging* portion of the plug between the fingers. The needle containing the bit of colony is now to be thrust into the medium in the tube if a stab-culture is desired, or rubbed gently over its surface if a smear or stroke-culture is to be made. The needle is then withdrawn, the cotton plug replaced, *and the needle sterilized* before it is laid down. Neither the needle nor its handle should touch the inner sides of the test-tube if it can be avoided. The tube is then labelled and set aside for observation. The growth which appears in the tube after twenty-four to thirty-six hours should be a pure culture of the organisms of which the colony was composed.

Cultures of this form are not only useful as a means of preserving the different organisms with which we may be working, but serve also to bring out certain characteristics of different organisms when grown in this way.

If gelatin be employed and the organism which has been introduced into it possesses the power of bringing about

liquefaction—*i. e.*, of digesting it—it will soon be discovered that the mode of liquefaction differs with different organisms and is practically constant for the same organism.

FIG. 32

Series of stab-cultures in gelatin, showing modes of growth of different species of bacteria.

Some bacteria cause a liquefaction which spreads across the whole upper surface of the gelatin and continues gradually downward; with others it occurs in a funnel-shape, the broad end of the funnel being uppermost and the point down-

ward, corresponding to the track of the needle; at times a stocking- or sac-like liquefaction may be noticed. (See Fig. 32.)

NOTE.—Obtain a number of organisms from different sources in pure cultures by the method given. Plant them as pure cultures, all at the same time, in gelatin—preferably gelatin of the same making—retain them under the same conditions of temperature, and sketch the finer differences in the way in which liquefaction occurs.

Select from your collection a non-spore-bearing, actively liquefying species. Cultivate it as a pure culture in nutrient bouillon for three days. Then heat this bouillon culture to 68° C. on a water-bath for ten minutes. In the meantime prepare several tubes containing each about 10 c.c. of:

Gelatin	7.00 grams
Phenol	0.25 gram
Water	100.00 c.c.

Let the carbolized gelatin in one tube remain solid, and bring that in another to a liquid state by gentle heat. On the surface of the gelatin in the first tube place 0.5 c.c. of the heated (and cooled) culture, and mark on the side of the tube the point of contact between the fluid culture and the solid gelatin. To the tube of liquefied gelatin add likewise 0.5 c.c. of the heated culture, mix it thoroughly with the gelatin, and place the tube containing the mixture in cold water until the mass becomes solid. Set both tubes aside at a temperature not above 20° C. Note what occurs at the end of an hour, by next day, and after three days. Alter the experiment by filtering the three-day-old bouillon culture through a porcelain or a Berkefeld filter, instead of heating it as directed above. Are the results modified? How do you interpret these results?

CHAPTER X.

Methods of Staining—Cover-slip Preparations—Impression Cover-slip Preparations—Solutions Employed—Preparation and Staining of Cover-slips—Staining Solutions—Special Staining Methods.

A COMPLETE list of solutions and methods that are recommended for the staining of bacteria is not essential to the work of the beginner, so that only those which are of the most common application will be given in this book. In general, it suffices to say that bacteria stain best with watery solutions of the basic aniline dyes, and of these, fuchsin, gentian-violet, and methylene-blue are those most frequently employed.

In practical work bacteria are either dried upon cover-slips and then stained, or stained in sections of tissues in which they have been deposited during the course of disease. In both processes the essential point to be borne in mind is that the bacteria, because of their microscopic dimensions, require to be more conspicuously stained than the surrounding materials upon the cover-slips or in the sections, otherwise their recognition is a matter of the greatest difficulty, if not of impossibility. For this reason, especially in section-staining, it frequently becomes necessary to decolorize the tissues after removing them from the staining-solutions, in order to render the bacteria more prominent, and for this purpose special methods, which provide for decolorization of the tissues without robbing the bacteria of their color, are employed. The ordinary method of cover-slip examination of bacteria, constantly in use in these studies, is performed in the following way:

COVER-SLIP PREPARATIONS.

In order that the distribution of the organisms upon the cover-slips may be uniform and in as thin a layer as possible it is essential that the slips should be clean and free from grease. For cleansing the slips several methods may be employed.

The simplest plan with new cover-slips is to immerse them for a few hours in strong nitric acid, after which they are rinsed in water, then in alcohol, then ether, and, finally, they may be kept in alcohol to which a little ammonia has been added. When about to be used they should be wiped dry with a clean cotton or silk handkerchief.

A method commonly employed is to remove all coarse adherent matter from slips and slides by allowing them to remain for a time in strong nitric or sulphuric acid. They are removed from the acid after several days, rinsed in water, and treated as above. Knauer suggests the boiling of soiled cover-slips and slides for from twenty to thirty minutes in a 10 per cent. watery solution of lysol, after which they are to be rinsed carefully in water until all trace of the lysol has disappeared. They are then to be wiped dry with a clean handkerchief.

Löffler's method, which provides for the complete removal of all grease, is to warm the cover-slips in concentrated sulphuric acid for a time and then rinse them in water, after which they are kept in a mixture of equal parts of alcohol and ammonia. They are to be dried on a cloth from which all fat has been extracted.

Steps in Making the Preparations.—Place upon the centre of one of the clean dry cover-slips a very small drop of water or physiological salt-solution. With a platinum needle,

which has been sterilized in a gas-flame *just before using* and allowed to cool, take up a very small portion of the colony to be examined and mix it carefully with the drop on the slip until there exists a very thin homogeneous film over the larger part of the surface. This is to be dried upon the slip by either allowing it to remain upon the table in the horizontal position under a cover, to protect it from dust, or by holding it *between the fingers (not with forceps)*, at some distance above a gas-flame, until it is quite dry. If held with the forceps over the flame at this stage, too much heat may be unconsciously applied, and the morphology of the organisms in the preparation distorted. When held between the fingers with the thin layer of bacteria *away from the flame* no such accident is likely to occur. When the whole pellicle is completely dried the slip is to be taken up with forceps, and, holding the side upon which the bacteria are deposited away from the direct action of the flame, it is to be passed through the flame three times, about a second being allowed for each transit. Unless the preliminary drying at the low temperature has been complete, the preparation will be rendered worthless by the subsequent "fixing" at the higher temperature, for the reason that the protoplasm of bacteria when moist coagulates at these temperatures, and in doing so the normal outline of the cells is distorted. If carefully dried before fixing, this does not occur and the morphology of the organism remains unchanged.

A better plan for the process of fixing is to employ a copper plate about 35 cm. long by 10 cm. wide by 0.3 cm. thick. This plate is laid upon an iron tripod and a small gas-flame is placed beneath one of its extremities. By this arrangement one can get a graduated temperature, beginning at the part of the plate above the gas-flame where it is

hottest, and becoming gradually cooler toward the other end of the plate, which may be of a very low temperature. By dropping water upon the plate, beginning at the hottest point and proceeding toward the cooler end, it is easy to determine the point at which the water just boils; it is at a little below this point that the cover-slips are to be placed, bacteria side up, and allowed to remain about ten minutes, when the fixing will be complete. In very particular comparative studies this plan is to be preferred to the process of passing the cover-slips through a flame, as the organisms are always subjected to the same degree of heat, and the distortions which sometimes occur from too great and irregular application of high temperatures may be eliminated. The fixing consists in drying or coagulating the gelatinous envelope surrounding the organisms, by which means they are caused to adhere to the surface of the cover-slip. It is sometimes desirable to fix the preparations without the use of heat, as in the case of pus or other exudates. In this event, after drying the thinly spread material carefully in the air, the cover-slip on which it is placed is immersed in a mixture of equal parts of absolute alcohol and ether for about 15 minutes. At the end of this time it may be removed and stained. The advantage of this method is that there is less distortion and, as a rule, less precipitation (or, perhaps better, no charring) of extraneous matter.

The majority of bacteria with which the beginner will have to deal stain readily with watery solutions of any of the basic aniline dyes, such, for instance, as fuchsin, methylene-blue, or gentian-violet.

To stain the fixed cover-slip preparation, it is taken by one of its edges between forceps, and a few drops of a watery solution of either of the dyes named are placed upon the

film and allowed to remain twenty to thirty seconds. The slip is then carefully rinsed in water, and without drying is placed *bacteria down* upon a slide; the excess of water is taken up by covering it with blotting-paper and gently pressing upon it, after which the preparation is ready for examination.

Another plan sometimes used is to bring the slip upon the slide, *bacteria down*, without rinsing off the staining-fluid; the excess of fluid is removed with blotting-paper and the preparation is ready for examination with the microscope. This method is satisfactory and time-saving, but must always be practiced with care. The staining-fluid should always be filtered before using, to rid it of insoluble particles which might be taken for bacteria.

If upon examination the preparation prove of particular interest, so that it is desirable to preserve it, then it may be mounted permanently. The drop of immersion oil is to be removed from the surface of the slip with blotting-paper, and the slip loosened, or rather floated, from the slide by allowing water to flow around its edges. It is then taken up with forceps, carefully deprived of the water adhering to it by means of blotting-paper, and allowed to dry. When dry it is mounted in xylol-Canada-balsam by placing a small drop of the balsam upon the surface of the film, and then inverting the slip upon a clean glass slide. It is sometimes desirable to have the balsam harden quickly, and a method that is commonly employed to induce this is as follows: the slide, held by one of its ends between the fingers, is warmed over a gas-flame until quite hot; a drop of balsam is then placed on the centre of it, and it is again warmed; the cover-slip is then placed in position, and when the balsam is evenly distributed the temperature is rapidly reduced

by rubbing the bottom of the slide with a towel wet with cold water. Usually the preparation is firmly fixed after this treatment; a little practice is necessary, however, in order not to overheat and crack the slide. The method is applicable only to cover-slip preparations, and cannot be safely used with tissues.

Impression Cover-slip Preparations.—Impression preparations differ from ordinary cover-slip preparations in only one respect: they present an impression of the organisms as they were arranged in the colony from which the preparation is made. They are made by gently covering the colony with a thin, clean cover-slip, *lightly* pressing upon it, and, without moving the slip laterally, lifting it by one of its edges. The organisms adhere to the slip in the same relation to one another that they had in the colony. The subsequent steps of drying, fixing, staining, and mounting are the same as those just given for ordinary cover-slip preparations.

By this method constancies in the arrangement and grouping of the individuals in a colony can often be made out. Some will always appear irregularly massed, others show growth in parallel bundles, while others, again, will be seen as long, twisted threads.

NOTE.—From a colony of *bacillus subtilis* make a cover-slip preparation in the ordinary way; now make an impression cover-slip preparation of another colony of the same organism. Compare the results.

ORDINARY STAINING-SOLUTIONS.

The solutions commonly employed in staining cover-slip preparations are, as has been stated, watery solutions of

the basic aniline dyes—fuchsin, gentian-violet, and methylene-blue. These solutions may be made either by directly dissolving the dyes in substance in water until the proper degree of concentration has been reached, or by using concentrated watery or alcoholic solutions of the dyes which may be kept on hand as stock. The latter method is the one commonly practised.

The solutions of the colors which are in constant use in staining are prepared as follows:

Prepare as stock, saturated alcoholic or watery solutions of fuchsin, gentian-violet, and methylene-blue. These solutions are best made by pouring into clean bottles enough of the dyes in substance to fill them to about one-fourth of their capacity. Each bottle should then be filled with alcohol or with water, tightly corked, well shaken, and allowed to stand for twenty-four hours. If by then all the staining-material has been dissolved, more should be added, the bottle being again shaken and allowed to stand for another twenty-four hours; this must be repeated until a permanent sediment of undissolved coloring-matter is seen upon the bottom of the bottle. The bottles are then to be labelled "saturated alcoholic" or "watery" solution of fuchsin, gentian-violet, or methylene-blue, as the case may be. *These alcoholic solutions are not directly employed for staining-purposes.*

The solutions with which staining is accomplished are made from the stock solutions by adding 5 c.c. of the latter to 95 c.c. of distilled water. These represent the staining-solutions in every-day use. They may be kept in bottles supplied with stoppers and pipettes (Fig. 33), and when used are dropped upon the preparation to be stained.

For certain bacteria which stain only imperfectly with

these simple solutions it is necessary to employ agents that will increase the penetrating action of the dyes. Experience has taught us that this can be accomplished by the addition to the solutions of small quantities of alkaline substances, or by dissolving the staining-materials in strong watery solutions of either aniline or carbolic acid, instead of water—in other words, by employing special solvents and mordants with the stains.

FIG. 33

Rack of bottles for staining-solutions.

Of the solutions thus prepared which may always be employed upon bacteria that show a tendency to stain imperfectly, there are three in common use—Löffler's alkaline methylene-blue solution; the Koch-Ehrlich aniline-water solution of either fuchsin, gentian-violet, or methylene-blue; and Ziehl's solution of fuchsin in carbolic acid. These solutions are as follows:

Löffler's alkaline methylene-blue solution:

Concentrated alcoholic solution of methylene-blue	30 c.c.
Caustic potash in 1:10,000 solution	100 c.c.

Koch-Ehrlich aniline water solution. To about 100 c.c. of distilled water aniline oil is slowly added, a few drops

at the time, until the solution has an opaque appearance, the vessel containing the solution being thoroughly shaken after each addition. It is then filtered through moistened filter-paper until the filtrate is clear. To 100 c.c. of the clear filtrate add 10 c.c. of absolute alcohol and 11 c.c. of the concentrated alcoholic solution of either fuchsin, methylene-blue, or gentian-violet, preferably fuchsin or gentian-violet.

Ziehl's carbol-fuchsin solution:

Distilled water	100 c.c.
Carbolic acid (crystallized)	5 grams
Alcohol	10 c.c.
Fuchsin in substance	1 gram

Or it may be prepared by adding to a 5 per cent. watery solution of carbolic acid the saturated alcoholic solution of fuchsin until a metallic lustre appears on the surface of the fluid.

The Koch-Ehrlich solution decomposes after a time, so that it is better to prepare it fresh in small quantities when needed than to employ old solutions. Solutions older than fourteen days should not be used.

The three solutions just given may be used for cover-glass preparations in the ordinary way.

In some manipulations it becomes necessary to stain the bacteria very intensely, so that they may retain their color when exposed to the action of decolorizing agents. These methods are usually employed when it is desirable to deprive surrounding objects or tissues of their color, in order that the stained bacteria may stand out in greater contrast. It is in these cases that the staining-solution with which the bacteria are being treated is to be warmed, and in some cases boiled, so as further to increase its penetrating action.

When so treated, certain of the bacteria will retain their color, even when exposed to very strong decolorizers. The tubercle bacillus is distinguished from the great majority of other bacteria by the tenacity with which it retains the color when treated in this way; it is an organism difficult to stain, but when once stained is equally difficult to rob of its color.

DECOLORIZING-SOLUTIONS.—As regards the employment of decolorizing-agents, it must always be borne in mind that objects which are easily stained are also easily decolorized, and those that can be made to take up the staining-material only with difficulty are also very difficult to rob of their color. The most common decolorizer in use is probably alcohol—not absolute alcohol, but alcohol containing more or less of water. Water alone has this property, but in a much less degree than dilute alcohol. On the other hand, a much more energetic decolorization than that possessed by either alone can be obtained by alternate exposures to alcohol and water. More energetic in their decolorizing action than either water or alcohol are solutions of the acids. They appear, particularly when they are alcoholic solutions, to diffuse rapidly into tissues and bacteria and very quickly extract the staining-materials which have been deposited there. For this reason these solutions should be employed with much care.

Very dilute acetic acid robs tissues and bacteria of their stain with remarkable activity; still more energetic are solutions of the mineral acids, and particularly, as has been said, when this action is accompanied by the decolorizing-properties of alcohol.

The acid solutions commonly employed are:

Acetic acid in from 0.1 to 5 per cent. watery solution.

Nitric acid in from 20 to 30 per cent. watery solution.

Sulphuric acid in from 5 to 10 per cent. solution in water.

Hydrochloric acid in from 1 to 3 per cent. solution in alcohol.

NOTE.—For details as to the technique of hardening and cutting sections and staining bacteria in tissues, the student is referred to Mallory and Wright's *Pathological technique*.

Method of Staining the Tubercle Bacillus.—Select from the sputum of a tuberculous subject one of the small, white, cheesy masses which it is seen to contain. Spread this upon a cover-slip, dry and fix it in the usual way. The slip is now to be taken by its edge with forceps and the film covered with a few drops of either the solution of Koch-Ehrlich or that of Ziehl. It is then held over a gas-flame, at first some distance away, gradually being brought nearer until the fluid begins to boil. After it has bubbled once or twice it is removed from the flame, the excess of stain washed away in a stream of water, then immersed in a 30 per cent. solution of nitric acid in water, and allowed to remain until all color has disappeared. This takes longer in some cases than in others. One can always determine if decolorization is complete by washing off the acid in a stream of water. If the preparation is still distinctly colored, it should be immersed again in the acid; if of only a very faint color, it may be dipped in alcohol, again washed in water, and stained with some contrast-color. If, for example, the tubercle bacilli have been stained with fuchsin, methylene-blue forms a good contrast-stain. In making the contrast-stain the steps in the process are exactly those followed in the ordinary staining of cover-slip preparations in general: the slip containing the stained tubercle bacilli is carefully rinsed in water, and a few drops of the methylene-blue

solution placed upon it and allowed to remain for thirty or forty seconds, when it is again rinsed in water and examined microscopically. For this purpose of observing the difference in behavior of the tubercle bacilli and the other organisms present in the preparation toward this method of staining, it is well to examine the preparation microscopically before the contrast-stain is made; then give it the contrast-color, and again examine. It will be seen that before the contrast-color has been given to the preparation the tubercle bacilli are the only stained objects to be made out, and the preparation appears devoid of other organisms; but upon examining it after it has received the contrast-color a great many other organisms will appear; these take on the second color employed, while the tubercle bacilli retain their original color. Before decolorization all organisms in the preparation were of the same color, but during the application of the decolorizing solution all except the tubercle bacilli gave up their color. This microchemical characteristic, together with other reactions to be described, serves to differentiate the tubercle bacillus from other organisms with which it might be confounded. A number of different methods have been suggested for the staining of tubercle bacilli, but the original method as employed by Koch is so satisfactory in its results that it is not advisable to substitute others for it. The above differs from the original Koch-Ehrlich method for the staining of tubercle bacilli in sputum only in the occasional employment of Ziehl's carbol-fuchsin solution and in the method of heating the preparation with the staining-fluid upon it.

As Nuttall has pointed out, however, the strong acid decolorizer used in this method can, with advantage, be replaced by much more dilute solutions, as a number of the

bacilli are entirely decolorized by the too energetic action of the strong acids. He recommends the following method of decolorization: after staining the slip or section in the usual way, pass it through three alcohols; it is then to be washed in a solution composed of

Water	150 c.c.
Alcohol	50 c.c.
Concentrated sulphuric acid	20 to 30 drops

From this it is removed to water and carefully rinsed. The remaining steps in the process are the same as those given in the other methods.

GABBETT'S METHOD for the staining of tubercle bacilli recommends itself because of its simplicity and the rapidity with which it can be performed. By many it is considered the best method for routine employment. It consists in staining the cover-slips, prepared in the manner given, for from two to five minutes in a cold carbol-fuchsin solution, after which they are subjected to the action of Gabbett's methylene-blue sulphuric acid solution. This latter consists of

Sulphuric acid (strength 25 per cent.)	100 c.c.
Methylene-blue, in substance	1 to 2 grams

The cover-slips are then rinsed in water and are ready for examination. The tubercle bacilli will be stained red by the fuchsin, while all other bacteria, cell-nuclei, etc., will be tinted blue.

Pappenheim's Decolorizer and Counter Stain.—As with the Gabbett method, the cover-slips are stained for from 5 to 10 minutes in cold carbol-fuchsin. They are then rinsed in water and kept, until they are of a pale blue color, in a decolorizing and counter-staining fluid made as follows:

To 100 c.c. of a saturated alcoholic solution of methylene blue add 1 gram of rosolic acid and 20 c.c. of glycerine. The bacilli are stained red, the balance of the field blue.

Gram's Method.—Another important differential method of staining which is very commonly employed is that recommended by Gram. In this method the objects are treated with an aniline-water solution of gentian-violet made after the formula of Koch-Ehrlich. After remaining in this for two or three minutes they are immersed in a solution composed of

Iodine	1 gram
Potassium iodide	2 grams
Distilled water	300 c.c.

In this they remain for about five minutes; they are then transferred to 95 per cent. alcohol and thoroughly rinsed.

This method is particularly useful in demonstrating the capsule which is seen to surround some bacteria, especially *micrococcus lanceolatus* of pneumonia.

After such treatment certain species of bacteria are found to be of a very dark purple color, while all else in the preparation is decolorized; other species lose their color entirely in the process. Those that retain the dark stain are commonly denominated as "Gram-positive" while those that lose their color are known as "Gram-negative." While the majority of bacteria are either definitely positive or negative to this reaction, there are a few species that are indeterminate in this particular, that is to say, they become partly decolorized and one cannot say certainly that they are either positive or negative. Under certain conditions of cultivation, and especially under conditions favorable to degenerative changes, some species that are normally

"Gram-positive" may in part or wholly lose their "Gram-positive" properties.

Two theories, one chemical the other physical, have been offered in explanation of the mechanism of the Gram method of staining. In the *chemical* theory it is believed that, through the intervention of the iodine, the gentian-violet is linked inseparably to the protoplasm of "Gram-positive" bacteria and is not so linked in the "Gram-negative" species. The *physical* theory assumes differences in permeability of either the bacterial envelope or the bacterial protoplasm. In those species that are highly permeable the precipitation resulting from the interaction between the iodine and the gentian-violet occurs so deeply within the bacterial structure that it is not readily washed out by the final alcohol bath, this would be the case with the "Gram-positive" species; while in the case of the "Gram-negative" species, assumed to be less permeable, the precipitation is upon their surfaces and is readily removed by the final rinsing in alcohol.

Glacial Acetic Acid Method.—Another method that may be employed for demonstrating the presence of the capsule surrounding certain organisms is to prepare the cover-slips in the ordinary way, then cover the layer of bacteria upon them with glacial acetic acid, which is instantly poured off (not washed off with water), and the aniline-water gentian-violet solution dropped upon them; this is allowed to remain three or four minutes, is poured off, and a few drops more are added, and lastly the slip is washed in a solution of sodium chloride of from 0.6 to 0.7 per cent. in strength; but at times it must be stronger, occasionally as concentrated as 1.5 to 2 per cent. The reason for this is that if the slips be washed in water, or in salt-solution that is too weak, the mucin capsule that has been

coagulated by the acetic acid is redissolved and rendered invisible. This does not occur when the salt-solution is of the proper strength—a point that can be determined only after a few trials with solutions of different strengths. (Welch.) A very clear, sharply cut picture usually follows this method of procedure.

Ribbert also recommends for the staining of capsulated bacteria the *momentary* immersion of the cover-slips in a saturated solution of dahlia in a mixture of 100 parts of water, 50 parts of alcohol, and $12\frac{1}{2}$ parts of glacial acetic acid; after which the excess of color is removed by washing in water.

Staining of Spores.—We have learned that one of the points by which spores may be recognized is their refusal to take up staining-substances when applied in the ordinary way. They may, however, be stained by special methods; of these, one that has given fairly satisfactory results in our hands is as follows: the cover-slip is to be prepared from the material containing the spores in the ordinary way, dried, and fixed. It is then to be held by its edge with forceps, and its surface covered with Löffler's alkaline methylene-blue solution. It is then held over the Bunsen flame until the fluid boils; it is then removed, and after a few seconds is heated again. This is continued for about one minute, after which it is washed in water and then decolorized in

Alcohol (80 per cent.)	98 c.c.
Nitric acid	2 c.c.

until all visible blue color has disappeared. It is then rinsed in water and dipped for from 3 to 5 seconds in

Saturated alcoholic solution of eosin	10 c.c.
Water	90 c.c.

after which it is again rinsed in water and finally mounted for examination. If the decolorization in the acid alcohol be not carried too far, the preparation will show the spores stained blue and the bodies of the cells to have taken on the rose color characteristic of eosin.

By another process the cover-slip is floated, bacteria down, upon the surface of freshly prepared Koch-Ehrlich solution of fuchsin contained in a watch-crystal. This is then held by its edge with forceps and moved up and down over a small Bunsen flame until the fluid boils gently. This is continued for 2 or 3 minutes. When the fluid has stood for about five minutes after boiling the preparation is transferred, without washing in water, to a second watch-crystal containing the following decolorizing solution:

Absolute alcohol	100 c.c.
Hydrochloric acid	3 c.c.

In this solution it is placed, bacteria up, and the vessel is tilted from side to side for about one minute. It is then removed, washed in water, and stained with the cold methylene-blue solution. The spores will be stained red and the body of the cells blue.

It must be remembered that there are conspicuous differences in the behavior of spores of different bacteria to staining-methods and of the spores of a single species in different stages of development. Some stain readily by either of the methods especially devised for this purpose, while others can hardly be stained at all, or only with the greatest difficulty, by any of the known processes; some stain readily when fully developed, but with difficulty when only partly developed; others have this peculiarity reversed.

Löffler's Method for Staining Flagella.—For the demonstration of the locomotive apparatus possessed by motile

bacteria we are indebted to Löffler. By a special method of staining, in which the use of mordants played the essential part, he has shown that these organisms possess very delicate, hair-like appendages, by the lashing movements of which they propel themselves through the fluid in which they are growing. The method as given by Löffler is as follows:

It is essential that the bacteria be evenly and *not too numerously* distributed upon the cover-slip. The slips must therefore be perfectly clean. (See *Löffler's method* of cleaning cover-slips.) Five or six of the carefully cleansed cover-slips are to be placed in a line on a table, and on the centre of each slip a very small drop of tap-water is placed. From the culture to be examined a minute portion is transferred to the first slip and carefully mixed with the drop of water; from this mixture a small portion is transferred to the second, and from the second to the third slip, and so on, in this way insuring a dilution of the number of organisms present in the preparations. These slips are then dried and fixed in the ordinary way. They are next to be warmed in the following solution:

Tannic acid solution in water (20 acid, 80 water)	. 10 c.c.
Cold saturated solution of ferrous sulphate	. 5 c.c.
Saturated watery or alcoholic solution of fuchsin	. 1 c.c.

This solution represents the mordant. A few drops of it are to be placed upon the film of bacteria on the cover-slip, which is then to be held over a flame until the solution begins to steam. *It should not be boiled.* After steaming, the mordant is washed off in water and finally in alcohol. The bacteria are then to be stained in a saturated aniline-water-fuchsin solution.

There are several points and slight modifications in con-

nection with this method that require to be emphasized in order to insure success: the culture to be employed should be young, not over 18–20 hours old; it should have developed for this time on fresh agar-agar at 37° to 38° C.; the mordant should not be perfectly fresh, as the best results are obtained from the use of old solutions that have stood exposed to the air and that have been filtered just before using; when placed on the cover-slip and held over the flame *never heat the mordant to the boiling-point; indeed, the best results are obtained when the preparation is held high above the flame and removed from it at the first evidence of vaporization, or, better still, a little before this point is reached.*¹

Duckwall's Method² is a modification of the Löffler method, and the results obtained thereby are very satisfactory.

Preparation of the Staining Agents.—The fixing agent is mordant, and the stain is carbol-gentian-violet or, preferably, carbol-fuchsin.

The Mordant.

Desiccated tannic acid	2 grams
Cold saturated solution ferrous sulphate (aqueous)	5 grams
Distilled water	12 c.c.
Saturated alcoholic solution of fuchsin	1 c.c.

The tannic acid is dissolved in the water first by the application of gentle heat, then the ferrous sulphate, and then the alcoholic solution of fuchsin are added. To these ingredients it is advisable to add from 0.5 to 1 c.c. of a 1 per cent. sodium hydroxide solution. The best grade of filter-paper is used for filtering the mordant, and there should be left a heavy precipitate. After filtering, the color

¹ I am indebted to Dr. James Homer Wright, Thomas Scott Fellow in Hygiene, 1892–1893, University of Pennsylvania, for some of the suggestions in connection with the modification of this method.

² The Canner, vol. xx, p. 23.

of this mordant should be of a reddish-brown hue, not clear, but somewhat cloudy, and this mordant must be used within five hours after it is made. After that time it loses its fixing power. This is indicated by its gradual clarification and darkened color. It gives the best results when strictly fresh, and accomplishes its work in a much shorter time, so that very little if any heating is required when it is placed on the cover-glass preparation.

The Stain.—To prepare the dye for this method take about 1 gram of ordinary granulated fuchsin, put it in a bottle, and pour over it about 25 c.c. of warm, absolute alcohol. Shake vigorously and let it stand for several hours before using. The carbol-fuchsin is made by diluting the saturated alcoholic solution four or five times with a 5 per cent. solution of carbolic acid. Carbol-fuchsin should be freshly made, heated, and filtered before using.

The application of this method of demonstrating the flagella varies with different organisms with regard to the length of time the mordant and stain are allowed to act, and the amount of sodium hydroxide solution used. Usually, it is well to heat the mordant on the cover-slip to steaming, and allow it to act from one-half to one minute. It is then washed off with water and a small quantity of alcohol poured over the surface and washed off instantly. The water on the cover-slip is now absorbed from the edge of the cover-slip with clean filter-paper. The carbol-fuchsin stain is now applied and heated just enough to generate a thin vapor. The stain should not act for more than from one-half to one minute. The cover-slip is now dried, then xylol is poured over the surface, the excess being removed with filter-paper. The cover-slip is now mounted in xylol balsam.

Stern's Method for the Staining of Spirochete Pallida.—The spiral organism discovered in the lesions of syphilis by Schaudinn and Hoffman, and generally regarded as the cause of syphilis, is most easily demonstrated by the following simple method:

Prepare cover-slips from the serous exudate of the syphilitic lesion. Allow them to dry, first at ordinary room temperature, then for an hour at 37° C. Do not heat over the flame. Immerse them in a 10 per cent. silver nitrate solution in a clear glass dish and expose to diffuse day light (*not direct sunlight*) for several hours until the films take on a brownish color and a metallic lustre. Then wash in water and mount for examination. The spirochete will be seen as almost black spirals lying in a more or less granular field.

CHAPTER XI.

Systematic Study of an Organism—Points to be Considered in Determining the Morphologic and Biologic Characters of a Culture—Methods by Which the Various Biologic and Chemical Characters of a Culture may be Ascertained—Dark Field Illumination—Facts Necessary to Permit the Identification of an Organism as a Definite Species.

AFTER isolating an organism in pure culture by the plate method, considerable work is necessary in order to establish its identity. Small portions of the pure culture are taken upon the point of a sterile platinum wire and transplanted into the various culture-media. These sub-cultures of the organism are then placed under suitable conditions of temperature and environment, and examined from day to day to note the alterations that occur in the different media. In the systematic study of an organism no one character can be relied upon to the exclusion of others. It is necessary to note the microscopic appearance of the individual organism and its behavior toward different staining solutions and other reagents; in addition it is necessary to note the gross appearance of the culture of the different media as shown by naked-eye (macroscopic) examination as well as under a lens of low magnifying power (microscopic); while equal importance must be given to the chemical alterations produced by the bacteria in the different media, and the influence of different reagents, when added to the media, to show the presence of certain metabolic products. In this manner the entire life history of an organism, outside the animal body, may be ascertained.

The different characters of an organism may be grouped as: (a) *morphologic*, those ascertained by examination of the individual organism under a lens of high magnifying power; (b) *biologic*, those ascertained by macroscopic and microscopic study of the gross appearance of the culture in the different media; (c) *biochemic*, the alterations produced in the different media as shown by direct examination or by the use of different reagents; and (d) *pathogenic*, the effects of the inoculation of the culture into susceptible animals.

SCHEME OF STUDY.—Record the source whence the organism was derived. Was this the normal habitat of the organism, or was it present accidentally?

MORPHOLOGIC CHARACTERS.

Note the shape, size, and grouping of the organism as it occurs in the different media. Observe the nature of the ends of the individual organism. Determine the presence or absence of motility in very young cultures. If motility is observed, apply one of the special methods for demonstrating flagella to note their relative number and location and do not be discouraged if your first attempts fail. Stain your cultures by means of the different staining solutions, and note the effect of each. Do the organisms stain deeply and uniformly, or are they stained in a peculiar manner? Apply the Gram method of staining, and note whether or not the organisms are decolorized by the alcohol. Stain the organisms deeply with carbol-fuchsin staining solution, and note the effect of different decolorizing agents; and ascertain whether the organisms are capable of resisting the decolorizing effects of dilute acids. Do the organisms show

the presence of a capsule when taken from the blood or tissues of an animal, or when taken from cultures in milk or blood-serum? Examine cultures that are several days old, and note whether spores are being formed. Note particularly the position of the spore within the cell. Is the spore of smaller or greater diameter than the cell in which it is forming? Examine cultures that are a week or more old, and note whether the organisms have undergone any definite alterations in form (involution forms), or whether they present evidences of fragmentation or granulation of their protoplasm (degeneration forms).

BIOLOGIC CHARACTERS.

Colony-formation.—Observe the character of the colonies formed in gelatin and agar-agar plates. Describe a typical surface colony and a typical deep colony, both as to their macroscopic and microscopic appearance. What is the relative size of the colonies formed on each of these media when they are sufficiently separated from one another to permit unhindered development? Note the color and internal structure of the colonies as well as their relative density. What is the nature of the surface contour and arrangement of the colonies? Note their general character, as to whether they are moist or dry, compact or loosely constructed, sharply circumscribed or spreading over the surface of the medium. Do the gelatin colonies show evidences of liquefaction?

Agar-slant Inoculations.—Observe the nature of the growth on the surface of an agar-agar slant inoculation. Describe the color, texture, and optical characters of the growth. Is the growth confined to the line of inoculation, or has it a

tendency to spread over the surface of the medium? Is it smooth or rough, moist or dry, glistening or dull in character? If the organism forms pigment, note whether the pigment is confined to the area of growth or whether it extends into the medium itself. Record the manner in which the culture changes in its appearance on successive days.

Agar-stab Inoculations.—Observe the nature of the growth in an agar-agar-stab inoculation. Note whether the growth is most voluminous at or near the surface or in the depth of the stab. If the organism produces pigment, note whether the pigment-formation is most marked at or near the surface or at the bottom of the stab. Record the alterations that are observed on several successive days.

Gelatin-stab Inoculations.—Observe the nature of the growth in a gelatin-stab inoculation. Is the growth most voluminous at or near the surface or at the bottom of the stab? Note the general character of the growth on the surface, especially as to its contour, extent, and color. Note the character of the growth in the stab. Is it continuous along the whole line of inoculation, or is it confined to isolated areas? If the organism has the property of liquefying gelatin, note carefully the manner in which the liquefaction proceeds. How soon does liquefaction begin, and in what length of time is a tube of gelatin completely liquefied?

Potato Culture.—Observe the nature of the growth on potato. This is an important differential medium, since some organisms grow very sparingly or without producing a visible growth. Other organisms grow very characteristically. Some organisms have the property of breaking up the starch of the potato into simpler compounds. This is sometimes accompanied by the evolution of gas. Many

of the chromogenic bacteria find the potato a most suitable pabulum on which to form their pigment, the pigment formed on this medium having at times an especial brilliancy. Note in detail all the changes that occur in the growth on successive days.

Growth in Bouillon.—Observe whether the fluid shows turbidity or not, as well as the extent and distribution of this alteration. Note whether any sediment is being formed, as well as the nature and amount of such sediment. Does the organism form a definite growth (pellicle or scum) on the surface of the bouillon? What is the character of the pellicle? Is it readily dislodged, and, when dislodged, is it replaced by a new pellicle? Note whether the color of the medium has become altered. Note the manner in which the appearance of the culture changes on several successive days.

Growth in Litmus-milk.—Observe the nature of the growth in litmus-milk. Has the reaction of the medium become altered? To what is such alteration attributable? Note whether there is precipitation of casein. Record the extent and rapidity with which this alteration takes place, as well as the reaction of the fluid while the change is being produced. Is there any evidence of the subsequent liquefaction of the precipitated casein? Has the litmus been altered in any manner except as shown by altered reaction of the medium? In what part of the tube has such alteration of the litmus commenced? If the litmus has been decolorized, is it possible to restore its color by the admixture of air with the fluid? Note the order in which the appearance of the medium changes on successive days.

Growth in Special Media.—The special culture-media may be employed to ascertain additional biologic characters of

an organism, such as the production of indol, reduction of nitrates to nitrites, the formation of ammonia, production of gas in media containing different carbohydrates, or the reducing power of the organism on aniline dyes, etc.

Influence of External Agencies.—Note the vitality of the organism under the influence of various physical and chemical agents. Determine the temperature at which it thrives best, as well as the lowest and highest temperatures at which growth is possible. Determine the thermal death-point of the organism by subjecting it to various degrees of temperature from 55° to 75° C. for ten minutes. Determine its resistance to drying; to the influence of light; to the influence of germicidal substances. Determine the influence of different gases upon the growth of the organisms, such as hydrogen, nitrogen, or carbon dioxide. Determine the chemical reaction of the culture-media best adapted for its growth.¹

BIOCHEMIC CHARACTERS.

If the organism exhibits chromogenic properties, ascertain whether the pigment is intra- or extracellular. Ascertain under what conditions of temperature, reaction, and constitution of media, or under what atmospheric conditions this function is best exhibited. Note the influence of different reagents upon the pigment, such as chloroform, ether, alcohol, water, acids, or alkalies. Note whether the organism exhibits photogenic properties, and if so, ascertain what conditions are most suitable for the manifestation of this phenomenon.

¹ For more detailed description of the variations in the character of the macroscopic and microscopic appearance of the cultures in the different media, the student is referred to Chester's *Determinative Bacteriology* and Eyre's *Bacteriologic Technique*.

Ascertain whether the organism produces enzymes. Does it manifest a proteolytic function, as shown by the liquefaction of gelatin, casein, or blood-serum? Note whether this function is manifested in alkaline or in acid condition of the medium. Does it manifest a precipitating effect (rennet ferment?) upon casein? Note whether this is manifested in alkaline or in acid condition of the medium. Does the organism have the property of breaking up any of the carbohydrates into simpler compounds? Is this alteration accompanied or not by the liberation of gas? If so, ascertain the relative amount of gas formed from a given quantity of carbohydrate. Analyze the gas formed, and state the relative proportion of carbon dioxide and residual (explosive) gas formed.

Ascertain whether the organism produces indol. Is this substance formed with the simultaneous reduction of nitrates to nitrites? Are the nitrites reduced further into ammonia?

PATHOGENIC PROPERTIES.

Ascertain whether any of the animals used for experimental purposes are susceptible when inoculated with the organism. Are all species of laboratory animals equally susceptible, or are some immune? Note the size of the dose and the manner of inoculation that gives the most constant and characteristic results. What are the symptoms and postmortem appearances produced? What is the location of the organisms in the body of the dead animal? Are they confined to the seat of inoculation, or are they distributed more or less generally throughout the body?

Note whether the virulence of the organism is maintained

when grown for several generations on artificial media, or whether it soon becomes attenuated. Which culture-medium is best suited to conserve the virulence of the organism? In what manner does its environment influence the virulence? If the virulence is readily lost, may it be regained by any of the known methods?

Ascertain whether the organism forms a soluble toxin when grown in fluid media, as sugar-free bouillon. If toxin is formed, ascertain whether the antitoxic state is readily induced in susceptible animals.

If no soluble toxin is formed, ascertain whether animals may be immunized by the injection of sub-lethal doses of dead or living cultures. Is a bactericidal immunity induced by this means? Does the serum of immune animals possess protective and curative properties when administered to susceptible animals before or after inoculation with the living organism? Does the serum of immune animals possess the property of agglutinating the organisms in relatively higher dilutions than the serum of normal animals of the same species?

The majority of the bacteria may be identified without resorting to such a detailed study of the biochemic and pathogenic properties as given in the foregoing outline, but for some of the pathogenic bacteria it has been necessary to apply all the known tests in order to definitely establish their identity. By means of such detailed studies on related organisms, it has been possible to differentiate varieties whose characters are constant, yet in general they are so closely related that it is impossible from the clinical manifestations produced to state definitely which particular variety of organism is responsible for the conditions. This

is especially true of the different varieties of bacillus dysenteriae, and of the group of typhoid and paratyphoid organisms. Further study will, no doubt, reveal variations in other pathogenic bacteria, which varieties are today regarded as distinct species.

MICROSCOPIC EXAMINATION OF PREPARATIONS.

The Different Parts of the Microscope.—Before describing the method of examining preparations microscopically, a few definitions of the terms used in connection with the microscope may not be out of place. (The different parts of the microscope referred to below are indicated by letters in Fig. 34.)

The *ocular* or *eye-piece* (A) is the lens at which the eye is placed when looking through the instrument. It serves to magnify the image projected through the objective.

The *objective* (B) is the lens which is at the distal end of the barrel of the instrument, and which serves to magnify the object to be examined.

The *stage* (C) is the shelf or platform of the microscope on which the object to be examined rests.

The *diaphragms* are the perforated stops that fit in the centre of the stage. They vary in size, so that different amounts of light may be admitted to the object by using diaphragms with larger or smaller openings.

The "*iris*" *diaphragm* (D) opens and closes like the iris of the eye. It is so arranged that its opening for admission of light can be increased or diminished by moving a small lever in one or another direction.

The *reflector* (E) is the mirror placed beneath the stage, which serves to illuminate the object to be examined.

The *coarse adjustment* (F) is the rack-and-pinion arrangement by which the barrel of the microscope can be quickly raised or lowered.

FIG. 34

The *fine adjustment* (G) serves to raise and lower the barrel of the instrument very slowly and gradually.

For the microscopic study of bacteria it is essential that the microscope be provided with an oil-immersion system and a sub-stage condensing apparatus.

The *oil-immersion* or *homogeneous system* consists of an objective so constructed that it can only be used when the transparent media through which the light passes in entering it are all of the same index of refraction—*i. e.*, are *homogeneous*. This is accomplished by interposing between the face of the lens and the cover-slip covering the object to be examined a body which refracts the light in the same way as do the glass slide, the cover-slip, and the glass of which the objective is made. For this purpose, a drop of oil of the same index of refraction as the glass is placed upon the face of the lens, and the examinations are made through this oil. There is thus little or no loss of light from deflection, as is the case in the dry system.

The *sub-stage condensing apparatus* (H) is a system of lenses situated beneath the central opening of the stage. They serve to condense the light passing from the reflector to the object in such a way that it is focussed upon the object, thus furnishing the greatest amount of illumination. Between the condenser and reflector is placed the “iris” diaphragm, the aperture of which can be regulated, as circumstances require, to permit of either a very small or a very large amount of light passing to the object.

The *nose-piece* (I) consists of a collar, or group of collars joined together (two or more), that is attached to the distal end of the tube of the microscope. It enables one to attach several objectives to the instrument in such a way that by simply rotating the nose-piece the various lenses of different power may be conveniently used in succession.

Dark-field Illumination.—This refers to a result obtained through the use of an apparatus that so deflects and reflects the light's rays that the field is dark and the objects in it brilliantly light. It is used only for the examination of unstained objects and is capable of revealing the most minute particles and micro-organisms. It is especially useful for the study of the normal morphology and movement of spirochete and for the detection of bodies so small or otherwise so constituted as not to be visible by the ordinary methods of microscopic examination. Two forms of the illuminator are in use—one that slips into the collar ordinarily carrying the substage condensing apparatus, the other is made in the form of a slide and is placed on the stage directly over the opening for illumination. Both provide for the complete cutting off of direct central rays of light, allowing only the lateral rays to reach the objects and be *reflected* by them to the eye. Both require very intense illumination for the best results. This may be obtained from a Welsbach burner, or a small arc light. In both cases the light's rays must be condensed upon the reflector of the microscope by means of a condensing lens.

Microscopic Examination of Cover-slips.—The stained cover-slip is to be examined with the oil-immersion objective, and with the diaphragm of the sub-stage condensing apparatus open to its full extent. The object gained by allowing the light to enter in such a large volume is that the contrast produced by the colored bacteria in the brightly illuminated field is much more conspicuous than when a smaller amount of light is thrown upon them. This is true not only for stained bacteria on cover-slips, but likewise for their differentiation from surrounding objects when they are located in tissues. With *unstained* bacteria and tissues, on the con-

trary, the structure can best be made out by reducing the bundle of light-rays to the smallest amount compatible with distinct vision, and in this way favoring, *not color-contrast*, but contrasts which appear as *lights and shadows*, due to the differences in permeability to light of the various parts of the material under examination.

Steps in Examining Stained Preparations with the Oil-immersion System.—Place upon the centre of the cover-slip which covers the preparation a small drop of immersion oil. Place the slide upon the centre of the stage of the microscope. With the coarse adjustment lower the oil-immersion objective until it *just touches* the drop of oil. Open the illuminating apparatus to its full extent. Then, with the eye to the ocular and the hand on the fine adjustment, turn the adjusting-screw toward *the right* until the field becomes somewhat colored in appearance. When this is seen proceed more slowly in the same direction, and, after one or two turns, the object will be in focus. *Do not remove the eye from the instrument until this has been accomplished.*

Then, with one hand upon the fine adjustment and the thumb and index finger of the other hand holding the slide lightly by its end, it may be moved about under the objective. At the same time the screw of the fine adjustment must be turned back and forth, so that the different planes of the preparation may be brought into focus one after the other. In this way the whole section or preparation may be inspected. When the examination is finished raise the objective from the preparation by turning the screw of the *coarse* adjustment *toward you*. Remove the preparation from the stage, and, with a fine silk cloth or handkerchief, *wipe very gently and carefully* the oil from the face of the lens.

During work, of course, the lens need not be cleaned and

put away after each examination; but when the work for the day is over an immersion lens is best protected in this way. Under no circumstances should it be allowed to remain in the immersion oil or exposed to dust for any length of time.

Examination of Unstained Preparations.—“*Hanging drops.*” It frequently becomes necessary to examine bacteria in the unstained condition. The circumstances calling for this arise while studying the multiplication of cells, the germination of spores, and the absence or presence of motility.

In this method the organisms to be studied are suspended in a drop of physiological salt solution or of bouillon, or a tiny drop of either agar-agar or gelatin, inoculated with the organism, may be employed. The drop is placed in the centre of a clean cover-slip which has been sterilized in the flame and which is then inverted over the depression in a sterilized so-called “hollow-ground” slide to which it is sealed with vaseline. A convenient and quick method of making the preparation is, after placing the drop in the centre of the cover-slip, to invert *over it* the slide, around the depression in which a ring of vaseline has been painted. The slip adheres and the preparation may then be handled without fear of disturbing the drop or the position of the slip over the depression. When completed it has the appearance shown in Fig. 35. The drop hangs in an air-tight chamber so that both evaporation and contamination are prevented.

This is known as the “hanging-drop” method of examination or cultivation. It is indispensable for the purposes mentioned, and at the same time requires considerable care in its manipulation. The fluid is so transparent that the cover-slip may be broken by the objective being brought

down upon the preparation before one is aware that the focal distance has been reached. This may be avoided by bringing the *edge* of the drop into the centre of the field with one of the higher power *dry* lenses. When this is accomplished substitute the immersion for the dry system, when the edge of the drop is readily detected with the higher power lens somewhere near the centre of the field.

In examining bacteria by this method there is a possibility of error that must be guarded against. All microscopic insoluble particles in suspension in fluids possess a peculiar tremor or vibratory motion, the so-called "Brownian motion." This is very apt to give the impression that the organisms under examination are motile, when in truth they

FIG. 35



Longitudinal section of hollow-ground glass slide for observing bacteria in hanging drops.

are not so, their movement in the fluid being only this molecular tremor.

The difference between the motion of bodies undergoing this molecular tremor and that possessed by certain living bacteria is that the former particles never move from their place in the field, while living motile bacteria alter their position in relation to the surrounding organisms, and may dart from one position in the field to another. In some cases the true movement of bacteria is very slow and undulating, while in others it is rapid and darting. The molecular tremor may be seen with non-motile and with dead organisms.

NOTE.—Prepare three hanging-drop preparations—one from a drop of dilute India-ink, a second from a culture of

micrococci, and a third from a culture of the bacillus of typhoid fever. In what way do they differ?

Study of Spore-formation.—The hanging-drop method just mentioned is not only employed for detecting the motility of an organism, but also for the study of its mode of spore-formation.

Since with aërobic organisms spore-formation occurs, as a rule, only in the presence of oxygen, and is induced more by limitation of the nutrition of the organisms than by any other factor, it is essential that these two points should be borne in mind in preparing the drop-cultures in which the process is to be studied. For this reason the drop of bouillon should be small and the air-chamber relatively large.

A very thin drop of sterilized agar-agar may be substituted for the bouillon. It serves to retain the organisms in a fixed position, and the process may be more easily followed.

As soon as finished the preparation is to be examined microscopically and the condition of the organisms noted. It is then to be retained in a warm chamber, and kept under continuous observation. The form of chamber best adapted to the purpose is one which envelops the whole microscope. It is provided with a window through which the light enters, and an arrangement by which the slide may be moved from the outside. The formation of spores requires a much longer time than the germination of spores into bacilli, but with patience both processes may be satisfactorily observed.

It will be noticed that the description of this process is very much like that which immediately precedes, but differs from it in one respect, viz., that in this manipulation we are not making a preparation which is simply to be examined and then thrown aside, but it is an actual pure

culture, and must be kept as such, otherwise the observation will be worthless. For this reason the greatest care must be observed in the sterilization of all objects employed. Studies upon spore-formation by this method frequently continue over hours, and sometimes days, and contamination must, therefore, be carefully guarded against. The study should be begun with the vegetative form of the organisms; the hanging-drop preparation should, for this reason, always be made from a perfectly fresh culture of the organism under consideration before time has elapsed for spores to form.

The simple detection of the presence or absence of spore-formation can in many cases be made by other methods. For example, many species of bacteria which possess this property form spores most readily upon media from which it is somewhat difficult for them to obtain the necessary nourishment; potatoes and agar-agar that have become a little dry offer very favorable conditions, because of the limited area from which the growing bacteria can draw their nutritive supplies, and because of the free access which they have to oxygen, for, their growth being on the surface, they are surrounded by this gas unless means are taken to prevent it. By the hanging-drop method, however, more than this specific property may be determined. It is possible not only to detect the stages and steps in the formation of endogenous spores, but when the spores are completely formed their germination into mature rods may be seen by transferring them to a fresh bouillon-drop or drop of agar-agar preserved in the same way. The word rods is used because we have as yet no evidence that endogenous spore-formation occurs in any of the other morphological groups of bacteria.

Hanging-block Cultures.—Hill¹ has devised a method for observing the development of individual bacteria, which consists in the substitution for the ordinary “hanging drop” of liquid or jelly a cube of solidified agar-agar, on the surface of which the bacteria are distributed.

The “hanging block” is prepared as follows: “Pour melted nutrient agar into a Petri dish to the depth of one-eighth to one-quarter inch. Cool this agar and cut from it a block about one-quarter to one-third inch square and of the thickness of the layer of agar in the dish. This block has a smooth upper and under surface. Place it, under surface down, on a slide and protect it from dust. Prepare an emulsion in sterile water of the organism to be examined if it has been grown on a solid medium, or use a broth culture; spread the emulsion or broth upon the upper surface of the block, as if making an ordinary cover-slip preparation. Keep the slide and block in an incubator at 37° C. for five to ten minutes to dry slightly. Then lay a clean sterile cover-slip on the inoculated surface of the block in close contact with it, avoiding, if possible, the formation of air-bubbles. Remove the slide from the lower surface of the block, and invert the cover-slip so that the agar-block is uppermost. With a platinum loop run a drop or two of melted agar along each side of the agar block where it is in contact with the cover-slip. This seal hardens at once, preventing slipping of the block. Place the preparation in the incubator again for five or ten minutes to dry the agar seal. Invert this preparation over a moist chamber and seal the cover-slip in place with white wax or paraffin. Vaseline softens too readily at 37° C., allowing shifting of the cover-slip. The preparation may then be examined at leisure.”

¹ Journal of Medical Research, 1902, vol. vii, p. 202.

Aërobic bacteria receive sufficient oxygen by diffusion, and for anaërobic bacteria it will suffice to hang the block in a chamber containing a little alkaline pyrogallic acid solution. This absorbs all oxygen.

Study of Gelatin Cultures.—As has been previously stated, the behavior of bacteria toward gelatin differs—some of them producing apparently no alteration in the medium, while the growth of others is accompanied by an enzymotic action that results in liquefaction of the gelatin at and around the place at which the colonies are growing. In some instances this liquefaction spreads laterally and downward, causing a saucer-shaped excavation; while in others the colony sinks almost vertically into the gelatin and may be seen lying at the bottom of a funnel-shaped depression. These differences are constantly employed as one of the means of differentiating otherwise closely allied species and varieties. (See Fig. 32.) Studies upon the spirillum of Asiatic cholera and a number of kindred species, for example, reveal decided differences in the form of liquefaction produced by these various organisms. The minutest detail in this respect must be noted, and its frequency or constancy under varying conditions determined.

Cultures on Potato.—A useful factor in the identification of an organism is its growth on sterilized potato. Many organisms present appearances under this method of cultivation which alone can almost be considered characteristic. In some cases coarsely lobulated, elevated, dry or moist patches of development occur after a few hours; again, the growth may be finely granular and but slightly elevated above the surface of the potato; at one time it will be dry and dull in appearance, again it may be moist and glistening. Sometimes bubbles, due to the fermentative action of

the growing bacteria on the carbohydrates of the potato, are produced.

A most striking form of development on potato is that often exhibited by the bacillus of typhoid fever and the bacillus of diphtheria. After inoculation of a potato with either of these organisms there is usually no naked-eye evidence of growth, though microscopic examination of scrapings from the surface of the potato reveals an active multiplication of the organisms which had been planted there. The potato is one of the first of the differential media.

CHANGES IN THE REACTION OF MEDIA AS A RESULT OF BACTERIAL ACTIVITY.

For purposes of differentiation, much stress is laid upon the reaction assumed by media as a result of bacterial growth. Under the influence of certain species the medium will become acid, under that of others it is alkaline, while some cause little or no change. In media of particular composition—*i. e.*, those containing traces of fermentable carbohydrates, notably muscle-sugar, as seen in infusions of fresh meat—the reaction may become acid with the beginning of growth and subsequently change to alkaline after the supply of fermentable sugar is exhausted. These changes of reaction are most conveniently observed through the use of indicators—bodies that either lose or change their usual color as the reaction of the medium to which they are added changes.

Such substances as litmus, in the form of the so-called “litmus tincture,” and coralline (rosolic acid) in alcoholic solution, are commonly employed for this purpose. They may be added to the media in the proportions given in the

chapter on Media, and the changes in their colors studied with different bacteria. Milk and litmus tincture or peptone solution to which rosolic acid has been added are excellent media for this experiment.

Fermentation.—The production of gas as an indication of fermentation is an accompaniment of the growth of certain bacteria. This is best studied in media to which 1 to 2 per cent. of grape-sugar (glucose) has been added. A convenient method of demonstrating this property is to employ a tube about half full of agar-agar containing the necessary amount of grape-sugar. The medium is to be liquefied on a water-bath, and then cooled to about 42° C., when a small quantity of a pure culture of the organism under consideration should carefully be distributed through it. The tube is then placed in ice-water and rapidly solidified in the vertical position. When solid it is placed in the incubator. After twenty-four to thirty-six hours, if the organism possesses the property of causing fermentation of glucose, the medium will be dotted everywhere with very small cavities containing the gas that has resulted.

This property of fermentation with evolution of gas is of such importance as a differential characteristic that considerable attention has been given to it, and those who have been most intimately concerned in the development of our knowledge on the subject do not consider it sufficient to say that the growth of an organism "is accompanied by the production of gas-bubbles," but that under given conditions we should determine not only the amount of gas or gases produced by the organism under consideration, but also their nature. For this purpose, Smith¹ recommends the

¹ An excellent and exhaustive contribution to this subject has been made by Theobald Smith in the *Wilder Quarter-Century Book*, Ithaca, N. Y., 1803.

employment of the fermentation-tube. This is a tube bent at an acute angle, closed at one end and enlarged with a bulb at the other. At the bend the tube is constricted. To it a glass foot is attached so that the tube may stand upright. (See Fig. 36.) To fill the tube, the fluid (it is used only with fluid media) is poured into the bulb until this is about half full. The tube is then tilted until the closed arm is nearly

FIG. 36

Fermentation-tube.

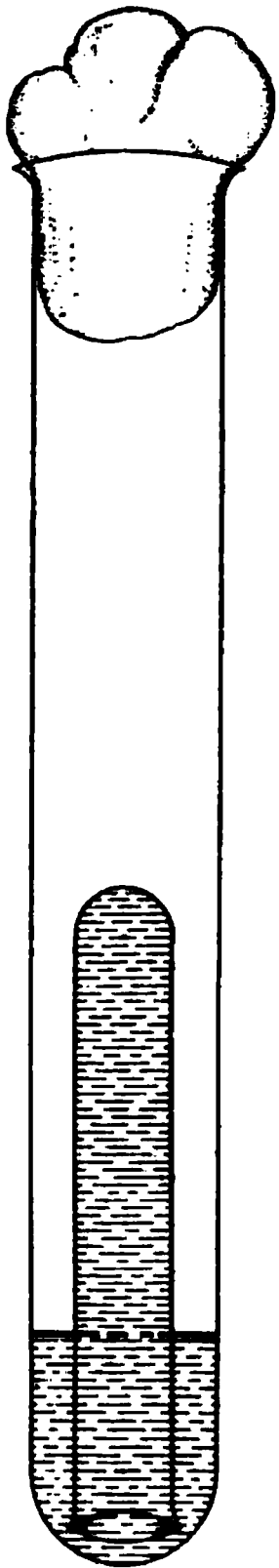
horizontal, so that the air may flow out into the bulb and the fluid flow into the closed arm to take its place. When this has been completely filled sufficient fluid should be added to bring its level within the bulb just beyond the bend, and the opening of the bulb plugged with cotton. The tubes thus filled are then to be sterilized. During sterilization they are to be maintained in the upright position. Under the

influence of heat the tension of the water-vapor in the closed arm forces most of the fluid into the bulb. As the tube cools, the fluid returns to its place in the closed arm and fills it again, with the exception of a small space at the top, which is occupied by the air originally dissolved in the liquid and which has been driven out by the heat. The air-bubble should be tilted out after each sterilization; and finally, after the third exposure to steam, this arm of the tube will be free from air. The medium employed is bouillon containing some fermentable carbohydrate, as glucose, lactose, or saccharose. After inoculation the flasks are placed in the incubator, and the amount of gas that collects in the closed arm is noted from day to day. From studies that have been made this gas is found to consist usually of about one part by volume of carbonic acid and two parts by volume of an explosive gas consisting largely of hydrogen. For determining the nature and quantitative relations of these gases Smith¹ recommends the following procedure: "The bulb is completely filled with a 2 per cent. solution of sodium hydroxide (NaOH) and closed tightly with the thumb. The fluid is shaken thoroughly with the gas and allowed to flow back and forth from bulb to closed branch and the reverse several times, to insure intimate contact of the CO₂ with the alkali. Lastly, *before removing the thumb all the gas is allowed to collect in the closed branch*, so that none may escape when the thumb is removed. If CO₂ be present, a partial vacuum in the closed branch causes the fluid to rise suddenly when the thumb is removed. After allowing the layer of foam to subside somewhat the space occupied by gas is again measured, and the difference between this amount and that measured before shaking with the sodium

¹ Loc. cit., p. 196.

hydroxide solution gives the proportion of CO_2 absorbed. The explosive character of the residue is determined as follows: the thumb is placed again over the mouth of the bulb and the gas from the closed branch is allowed to flow into the bulb and mix with the air there present. The plug is then removed and a lighted match inserted into the mouth of the bulb. The intensity of the explosion varies with the amount of air present in the bulb."

FIG. 37



Durham's fermentation-tube.

Durham's Fermentation-tube.—Durham employs a convenient modification of the ordinary fermentation-tube, which is constructed in the following manner: test-tubes of about 10 or 12 c.c. capacity are placed in an inverted position within a larger test-tube, and the latter plugged with cotton in the usual way and sterilized. (See Fig. 37.) The small tube should fit loosely within the larger one. The medium to be used is run into the larger tube until there is present about 50 per cent. more than the volume of the smaller tube. The whole is then sterilized in streaming steam by the fractional method. After the first sterilization the small tube will be found almost filled with fluid, over which a small air-bubble lies. After the second or third sterilization this

air-bubble is completely expelled, and the small tube contains nothing but the liquid.

The medium that Durham employs for the fermentation-test is a 1 per cent. solution of Witte's peptone in distilled water, to which have been added known amounts of some such fermentable sugar as glucose, saccharose, lactose, mannite, etc., as the case may demand. He prefers peptone to meat-infusion bouillon for the reason that the latter often contains traces of muscle-sugar, and is thereby likely to complicate the results. He prefers neutralization with organic acids rather than mineral acids, and uses citric acid by preference, the reason for this being that where sugars such as those mentioned are acted upon by mineral acids under the influence of heat their composition is apt to be altered.

NOTE.—Prepare two fermentation-tubes as follows: Fill one with 1 per cent. watery solution of peptone to which 2 per cent. of glucose has been added; fill the other with a similar peptone solution, but to which only 0.3 per cent. of glucose has been added. Sterilize and inoculate with *bacillus coli communis*. How do the two tubes differ from one another after eighteen to twenty-four hours in the incubator? First, as regards the reaction of the fluid in the open arms of the tubes. Second, as to accumulation of gas in closed arms of the tubes. Third, as to the capacity of each solution for reducing copper in Fehling's solution. What differences are observed, and how may they be explained?

Indol Production.—The detection of products other than those that give rise to alterations in the reaction of the media, and whose presence may be demonstrated by chemical

reactions, is a routine step in the identification of different species of bacteria. Among these bodies is one that is produced by a number of organisms, and whose presence may easily be detected by its characteristic behavior when treated with certain substances. I refer to *nitroso-indol*, the reactions of which were described by Beyer in 1869, and the presence of which as a product of the growth of certain bacteria has since furnished a topic for considerable discussion.

Indol, the name by which this body is generally known, when acted upon by reducing-agents becomes of a more or less decided rose color. This body was recognized as one of the products of growth of the spirillum of Asiatic cholera first by Poel, and a short time subsequently by Bujwid and by Dunham, and for a time was believed to be peculiarly characteristic of the growth of this organism. It has since been found that there are many other bacteria which also possess the property of producing indol in the course of their development. It is constantly present in putrefying matters, and is one of the aromatic compounds that give to feces their characteristic odor.

The methods employed for its detection are as follows: cultivate the organism for twenty-four to forty-eight hours at a temperature of 37° C., in the simple peptone solution known as "Dunham's solution" (see formula for this medium). This solution is preferred because its pale color does not mask the rose color of the reaction when the amount of indol present is very small.

Four tubes should always be inoculated and kept under exactly the same conditions for the same length of time.

At the end of twenty-four or forty-eight hours the test may be made. Proceed as follows: to a tube containing

7 c.c. of the peptone solution, but which has *not* been inoculated, add 10 drops of concentrated sulphuric acid. To another similar tube add 1 c.c. of a 0.01 per cent. solution of sodium nitrite, and afterward 10 drops of concentrated sulphuric acid. Observe the tubes for five to ten minutes. No alteration in their color appears, or at least there is no production of a rose color. They contain no indol.

Treat in the same way, with the acid alone, two of the tubes which *have been inoculated*. If no rose color appears after five or ten minutes, add 1 c.c. of the sodium nitrite solution. If now no rose color is produced, the indol reaction may be considered as negative—*i. e.*, no indol has been formed as a product of the growth of the bacteria.

If indol is present, and the rose color appears after the addition of the acid alone, it is plain that not only indol has been formed, but coincidently a reducing-body. This is found, by proper means, to be nitrous acid. The sulphuric acid liberates this acid from its salts and permits of its reducing action being brought into play.

If the rose color appears only after the addition of both the acid and the nitrite solution, then indol has been formed during the growth of the organisms, but no nitrites.

Control the results obtained by treating the two remaining cultures in the same way.

The test is sometimes made by allowing concentrated sulphuric acid to flow down the sides and collect at the bottom of the tube; the reaction is then seen as a rose-colored zone overlying the line of contact of the acid and culture-medium. This method is open to the objection that, if indol is present in only a very small amount, the faint rose tint produced by it is apt to be masked by a brown color that results from the charring action of the concentrated

acid on the other organic matters in the culture-medium, so that its presence may in this way escape detection. In view of this, Petri recommends the use of *dilute* sulphuric acid. He states that when indol is present the characteristic rose color appears a little more slowly with the dilute acid, but it is more permanent, and there is never any likelihood of its presence being masked by other color-reactions.

Muir and Ritchie recommend the use of ordinary fuming or yellow nitric acid for this test. In this method two or three drops of the acid are added to the culture under consideration. If indol be present, the red color appears as a result of the reducing action of the nitrous acid upon it. The defect in this method is that it reveals only the presence of indol, and fails to indicate whether or not reducing-bodies were coincidentally formed with the indol. As a test for indol alone it is convenient and entirely trustworthy.

Reducing Power of Bacteria.—The power to reduce chemical compounds from a higher to a lower state may be said to be common to all bacteria. In some bacteria, perhaps the majority, it is most conspicuously manifested in connection with substances containing sulphur, hydrogen sulphide being formed. In other bacteria it is best seen in connection with the alterations produced in certain pigments, as litmus, methylene-blue, indigo, etc., the normal color disappearing in part or entirely according to the nature and activity of the process. Other bacteria have the property of reducing certain salts, as in the reduction of nitrates to nitrites, or even to ammonia by the denitrifying bacteria. In some instances these reductions result from the fact that the bacteria liberate hydrogen from the compounds, in others it results from the fact that the bacteria abstract oxygen from such compounds, while in still other instances the

reduction is of a more complex nature. Each of these changes, therefore, indicates the nature of some of the metabolic activities manifested by the bacteria in question.

Test for Hydrogen Sulphide.—The reduction of sulphur compounds may be determined by growing the bacteria in peptone solution containing ferric tartrate, when the presence of hydrogen sulphide will be indicated by the brownish-black or jet-black color of the precipitated iron-sulphide.

Reduction of Nitrates.—The complete reduction of nitrates is brought about by many bacteria. Other bacteria are capable of carrying the reducing action as far as the formation of ammonia, while still others merely reduce the nitrates to nitrites. These reducing functions are encouraged and may be demonstrated by cultivating the bacteria in peptone solution containing potassium nitrate.

Test for Nitrites.—The method of Griess, as modified by Ilosvay, is quite satisfactory. These reagents are required:

(a) Naphthylamine	0.1 gram
Distilled water	20.0 c.c.
Acetic acid (25 per cent. solution)	150.0 c.c.
(b) Sulfanilic acid	0.5 gram
Acetic acid (25 per cent. solution)	150.0 c.c.

In preparing solution *a* the naphthylamine is dissolved in 20 c.c. of boiling water, filtered, allowed to cool, and mixed with the dilute acetic acid. Solutions *a* and *b* are then mixed. It is best prepared as needed, though it may be preserved for some time in a glass-stoppered bottle.

In testing for nitrites the reagent is added in the proportion of one volume of reagent to five volumes of culture. When nitrites have been formed a deep-red color appears in a few seconds. If no nitrites have been formed the culture remains colorless. In testing cultures it is always necessary to control

the results by blank tests on a portion of the same medium that had not been inoculated, as some of the ingredients of the medium may have contained nitrites.

Another test for the formation of nitrites is a mixture of starch and potassium iodide, as follows:

Starch	2.0 grams
Potassium iodide,	0.5 gram
Water	100.0 c.c.

Warm the mixture until the starch is completely dissolved. In testing for nitrites add 0.5 c.c. of the reagent to a tube of culture, and follow this by the addition of 2 or 3 drops of pure sulphuric acid. If nitrites have been formed, a dark-blue or purple color will appear. Control-tubes of the medium show no color reaction, or merely a trace of blue coloration.

Test for Ammonia.—The formation of ammonia may be detected by testing with Nessler’s reagent. The most satisfactory results are obtained by cultivating the organisms in a litre of culture fluid and then distilling off portions of the culture, collecting in Nessler tubes, and applying 1 c.c. of the reagent to each 50 c.c. of the distillate. The presence of ammonia in the distillate is shown by the yellow coloration resulting from the addition of the reagent.

The direct application of the reagent to the culture will give satisfactory results if a great deal of ammonia has been formed. In this instance the mercury in the reagent will be precipitated as mercurous oxide. Another rough test for the formation of ammonia is to place a strip of filter-paper—moistened with the Nessler reagent—over the mouth of a test-tube containing the culture, and then gently heating the culture. As the ammonia is driven off by the heat, it will react on the reagent on the strip of paper.

Examination of Cultures for Bacterial Toxins.—In the systematic study of a pathogenic organism it is necessary to know whether it is capable of producing a soluble toxin when growing in culture-media. This is done by filtering cultures of various ages and testing the effect of the filtrate upon susceptible animals.

FILTRATION OF CULTURES.—A variety of filters have been devised for the purpose of filtering liquid cultures and other fluids to obtain sterile filtrates. These filters are usually constructed of unglazed porcelain or of infusorial earth, and are made in the form of hollow cylinders or bulbs. The best-known forms of bacterial filters are the Chamberland and the Berkefeld. All the filters used for this purpose require some motive power to force the fluid through the filter. Compressed air may be employed to force the fluid through the filter, or atmospheric pressure may be utilized by creating a negative pressure on the distal side of the filter by the use of an air-pump.

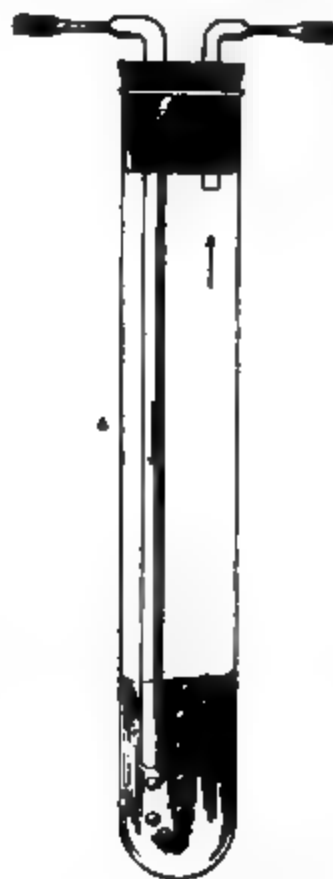
It is always necessary to test the sterility of the filtrate by making cultures from it into nutritive media and noting whether growth takes place or not.

Cultivation without Oxygen.—As we have already learned, there is a group of bacteria to which the designation “anaërobic” has been given, which are characterized by inability to grow in the presence of free oxygen. For the cultivation of the members of this group, a number of devices are employed for the exclusion of free oxygen from the cultures.

Method of Buchner. The plan suggested by Buchner, of allowing the cultures to develop in an atmosphere robbed of its oxygen by pyrogalllic acid, gives very good results. In this method the culture, which is either a slant- or stab-culture in a test-tube, is placed—tube, cotton plug, and all—

into a larger tube, in the bottom of which have been deposited 1 gram of pyrogallic acid and 10 c.c. of $\frac{1}{10}$ normal caustic-potash solution. The larger tube is then tightly plugged with a rubber stopper. The oxygen is quickly absorbed by the pyrogallic acid, and the organisms develop in the

FIG. 38



Fränkel's method for the cultivation of anaerobic bacteria.

remaining constituents of the atmosphere, viz., nitrogen, a small amount of CO_2 , and a trace of ammonia.

Method of C. Fränkel. Carl Fränkel suggested the following: the tube is first inoculated as if it were to be poured as a plate or rolled as an ordinary Esmarch tube. The cotton plug is then replaced by a rubber stopper, through which

pass two glass tubes. These must all have been sterilized in the steam sterilizer before using. On the outer side of the stopper these two tubes are bent at right angles to the long axis of the test-tube into which they are to be placed, and both are slightly drawn out in a gas-flame. Both of these tubes must be provided, before sterilization, with a plug of cotton; this is to prevent the access of foreign organisms to the medium during manipulations. At the inner side of the rubber stopper—that is, the end which is to be inserted into the test-tube—the glass tubes are of different lengths: one reaches to within 0.5 cm. of the bottom of the test-tube, the other is cut off flush with the under surface of the stopper. The outer end of the longer glass tube is then connected with a hydrogen generator and hydrogen is allowed to bubble through the gelatin (Fig. 38, A) in the tube until all contained air has been expelled and its place taken by the hydrogen.¹ When the hydrogen has been bubbling through the gelatin for about five minutes (at least) one can be reasonably sure that all oxygen has

¹ Before beginning the experiment it is always wise to test the hydrogen—i. e., to see that it is free from oxygen and that there is no danger of an explosion, for unless this be done the entire apparatus may be blown to pieces and a serious accident occur. The agents used should be pure zinc and pure sulphuric acid of about 25 to 30 per cent. strength. With the primary evolution of the gas the outlet of the generator should be closed and kept closed until the gas reservoir is quite filled with hydrogen. The outlet should then be opened and the entire volume of gas allowed to escape, care being taken that no flame is in the neighborhood. This should be repeated, after which a sample of the hydrogen generated should be collected in an inverted test-tube in the ordinary way for collecting gases over water, viz., by filling a test-tube with water, closing its mouth with the thumb, inverting it, and placing its mouth under water, when, after removing the thumb, the water will be kept in it by atmospheric pressure. The hydrogen which is flowing from the open generator may be conducted to the test-tube by rubber tubing. When the water has been replaced test the gas by holding a flame near the open mouth of the test-tube. If no explosion occurs, the hydrogen is safe to use. Should there be an explosion, the generation of hydrogen must be continued in the apparatus until it burns with a colorless flame when tested in a test-tube.

been expelled. The drawn-out portions of the tubes can then be sealed in the gas-flame without fear of an explosion. The protruding end of the rubber stopper is then painted around with melted paraffin and the tube rolled in the way given for ordinary Esmarch tubes. A tube thus prepared and containing growing colonies is shown in Fig. 38, *B*.

The development that now occurs is in an atmosphere of hydrogen, all oxygen having been expelled. During the operation the tube containing the liquefied gelatin should be kept in a water-bath at a temperature sufficiently high to prevent its solidifying, and at the same time not high enough to kill the organisms with which it has been inoculated.

One of the obstacles to the successful performance of this method is the bubbling of the gelatin, the foam from which will often fill the exit-tube and sometimes be forced from it. This may be obviated by reversing the order of proceeding, viz.: roll the Esmarch tube in the ordinary way with the organisms to be studied, using a relatively small amount of gelatin, so as to have as thin a layer as possible when it is rolled. Then replace the cotton plug with the sterilized rubber stopper carrying the glass tubes through which the hydrogen is to be passed, and allow the hydrogen to flow through as in the method first given. The gas now passes *over* the gelatin instead of *through* it, and consequently no bubbling results. In all other respects the procedure is the same as that given by Fränkel.

Method of Kitasato and Weil.—For favoring anaërobic conditions Kitasato and Weil have suggested the addition to the culture-media of some strong reducing-agent. They recommend formic acid or sodium formate in 0.3 to 0.5 per cent.; glucose in 1.5 to 2 per cent.; or blue litmus tincture in 5 per cent. by volume. This is, of course, in addition to

an atmosphere from which all oxygen has been expelled. As a reducing-agent for this purpose, Theobald Smith regards a weaker solution of glucose, 0.3 to 0.5 per cent., as more advantageous; and Wright obtains better results when glucose is added if the primary reaction of the media is about neutral to phenolphthalein.

Method of Park. A very simple, convenient, and efficient method is employed by Park. It consists in covering the medium in which the anaërobic species are to be cultivated with liquid paraffin (albolene). The best results are obtained when the amount of paraffin added is about half that of the liquid in the tube or flask. The liquid paraffin has the advantage over the solid paraffin in not retracting from the walls of the vessel on cooling. All air is expelled from flasks or tubes prepared in this way, by heating them in the autoclave. The layer of paraffin prevents the reabsorption of oxygen driven off by the heat. After cooling, the inoculation is made by passing the needle through the paraffin well down into the media.

Many other methods are employed for this special purpose, but for the beginner those given will suffice.

From what has been said, it may be inferred that the cultivation of anaërobic bacteria is a simple matter attended with but little difficulty. Such an opinion will, however, be quickly abandoned when the beginner attempts this part of his work for the first time, and particularly when his efforts are directed toward the separation of these forms from other organisms with which they are associated. The presence of spore-forming, *facultative* anaërobes in mixed cultures is always to be suspected, and it is this group that renders the task so difficult. At best the work requires undivided attention and no small degree of skill in bacteriological technique.

CHAPTER XII.

Inoculation of Animals—Subcutaneous Inoculation—Intravenous Injection—Inoculation into the Lymphatic Circulation—Inoculation into the Great Serous Cavities, and into the Anterior Chamber of the Eye—Observation of Animals after Inoculation.

AFTER subjecting an organism to the methods of study that we have thus far reviewed there remains to be tested its action on animals—*i. e.*, to determine if it possesses the property of producing disease or not; and, if so, what are the pathological results of its growth in the tissues of animals, and in what way must it gain entrance to the tissues in order to produce those results? The mode of deciding these points is by inoculation, which is practised in different ways according to circumstances. Most commonly a bit of the culture to be tested is simply deposited beneath the skin of the animal; but in other cases it may be necessary to introduce it directly into the vascular or lymphatic circulation, or into one or the other of the great serous cavities; or, for still other purposes of observation, into the anterior chamber of the eye, upon the iris or within the skull cavity, upon the dura or brain substance.

SUBCUTANEOUS INOCULATION OF ANIMALS.

The animals usually employed in the laboratory for purposes of inoculation are white mice, gray house-mice, guinea-pigs, rabbits, and pigeons.

For simple subcutaneous inoculation the steps in the

process are practically the same in all cases. The hair or feathers are to be carefully removed. If the skin is very dirty, it may be scrubbed with soap and water. Sterilization of the skin is practically impossible, so it need not be attempted. If the inoculation is to be made by means of a hypodermic syringe, then a fold of the skin may be lifted up and the needle inserted in the usual way. If a solid culture is to be inoculated, a fold of skin may be taken up with forceps and a tiny pocket cut into it with scissors which have previously been sterilized. This pocket must be large enough to admit the end of the needle without its touching the sides of the opening as it is inserted. Beneath the skin will be found the superficial and deep connective-tissue fasciæ. These must be taken up with sterilized forceps, and with sterilized scissors incised in a way corresponding to the opening in the skin. The pocket is then to be held open with the forceps and the substance to be inserted is introduced as far under the skin and fasciæ as possible, care being taken not to touch the edges of the wound if it can be avoided. The edges of the wound may then be simply pulled together and allowed to remain. No stitching or efforts at closing it are necessary, though a drop of collodion over the point of operation may serve to lessen contamination.

As the subcutaneous inoculation is very simple and takes only a few moments, guinea-pigs, rabbits, and pigeons may be held by an assistant. The front legs in the one hand and the hind legs in the other, with the animal stretched upon its back on a table, is the usual position for the operation when practised upon guinea-pigs and rabbits. The point at which the inoculations are commonly made is in the abdominal wall, either to the right or left of the median

line and about 3 cm. distant. When pigeons are used they are held with the legs, tail, and ends of the wings in the one hand, and the head and anterior portion of the body in the other, leaving the area occupied by the pectoral muscles, over which the inoculation is to be made, free for manipulation. In the case of fur-bearing animals the hair over the point selected for the inoculation should be closely cut with scissors, and from a small area the feathers should be plucked in the case of birds.

FIG. 39

Kitasato's mouse-holder.

It is at times, however, more convenient to dispense with an assistant; one of several forms of apparatus that have been devised for holding mice, guinea-pigs, rats, rabbits, etc., may then be used. For small animals, such as mice and rats, the holder suggested by Kitasato is very useful. It is simply a metal plate attached to a stand by a clamped ball-and-socket joint, so that it can be fixed in any position. It is provided with a spring-clip at one end that holds the

animal by the skin of the neck, and at the other end with another clamp that holds the tail of the animal. This holder is shown in Fig. 39. For larger animals the form of holder shown in Fig. 40 is commonly used.

The holder devised by Sweet,¹ which can be made of any size, from that suitable to a guinea-pig up to that large enough to secure a dog, is in every way the most convenient that we have encountered and, from the standpoint of the animal, is the most humane. It consists of four pieces of heavy round wire so bent that two engage the animal

FIG. 40

Holder for larger animals.

immediately behind the lower jaw while the remaining two close over the muzzle. All are held in position by a single clamp controlled by a single thumb-screw. When the screw is reversed and the clamp opened the anterior and posterior wire of each pair falls away from the median line, thereby liberating the animal. To secure the animal it is placed upon its back, the head laid in the cradle formed by the bent wires, the latter are adjusted to the proper position,

¹ A Simple, Humane Holder for Small Animals Under Experiment, University of Penna. Med. Bull., 1903, No. 2, p. 78.

and all secured by the turn of the single set-screw. Of course, the extremities of the animal are to be secured. This is done by means of cords securely held by a patent fastener made by the Tie Co., of Unadilla, N. Y. These fasteners are in every way more convenient than the cleats in common use. An idea of the apparatus is given in Fig. 41.

FIG. 41



A very simple and useful holder for guinea-pigs consists of a metal cylinder of about 5 cm. diameter and about 13 cm. long, closed at one end by a perforated cap of either tin or wire netting. Along the side of this box is a longitudinal slit 12 mm. wide that runs for 9.5 cm. from within 0.5 cm. of the open extremity of the cylinder. The animal is placed

in such a cylinder with its head toward the perforated bottom. It is then easily possible to make subcutaneous inoculation by taking up a bit of skin through the slit in the

FIG. 42

The Voges holder for guinea-pigs.

side of the box, or to make intraperitoneal injection by drawing the posterior extremities slightly from the box and holding them steady between the index and second finger, as seen in Fig. 42. It is also very convenient for use when the

rectal temperature of these small animals is to be taken. The manipulation can easily be made without the aid of an assistant. Its construction is seen in Fig. 42.¹

For ordinary subcutaneous inoculations at the root of the tail in mice a simple apparatus consists of a piece of board about 7 x 10 cm. and 2 cm. thick, upon which is tacked a hollow truncated cone of wire gauze, about 6 cm. long and about 1.5 cm. in diameter at one end and 2 cm. at its other end. This is tacked upon the board in such a position that its long axis is in the long axis of the board, being equidistant from its sides. Its small end is placed at the edge of the

FIG. 43

Mouse-holder, with mouse in proper position.

board. The mouse is taken up by the tail by means of a pair of tongs and allowed to crawl into the smaller end of the wire cone. When so far in that only the root of the tail projects the animal is fixed in this position by a clamp and thumb-screw, with which the apparatus (Fig. 43) is provided. The animal usually remains perfectly quiet and may be handled without difficulty.

The hair over the root of the tail is to be carefully cut away with scissors and a pocket cut through the skin at this point. The inoculation is then made into the loose

¹ *Centralblatt für Bacteriologie and Parasitenkunde*, 1895, vol. xviii, p. 530.

tissue under the skin over this part of the back in the way that has just been described. It is always best to insert the needle some distance along the spinal column, and thus deposit the material as far from the surface-wound as possible.

Injection into the Circulation.—It is not infrequently desirable to inject the material under consideration directly into the circulation of an animal. If a rabbit is employed for the purpose, the operation is usually done upon one of the veins in the ear. To those who have had no practice with this procedure it offers a great many difficulties; but if the directions which will be given are strictly observed the greatest of these obstacles to the successful performance of the operation may be overcome.

When viewing the circulation in the ear of the rabbit by transmitted light three conspicuous branches of the main vessel (*vena auricularis posterior*) will be seen. One runs about centrally in the long axis of the ear, one runs along its anterior margin, and one along its posterior margin. The central branch (*ramus anterior* of the *vena auricularis posterior*) is the largest and most conspicuous vessel of the ear, and is, therefore, believed by the inexperienced to be the branch into which it would appear easiest to insert a hypodermic needle. This, however, is fallacious. This vessel lies very loosely imbedded in connective tissue, and, in efforts to introduce a needle into it, rolls about to such an extent that only after a great deal of difficulty does the experiment succeed. On the other hand, the posterior branch (*ramus lateralis posterior* of the *vena auricularis posterior*) is a very fine, delicate vessel which runs along the posterior margin of the ear, and is so firmly fixed in the dense tissues which surround it that it is prevented from rolling

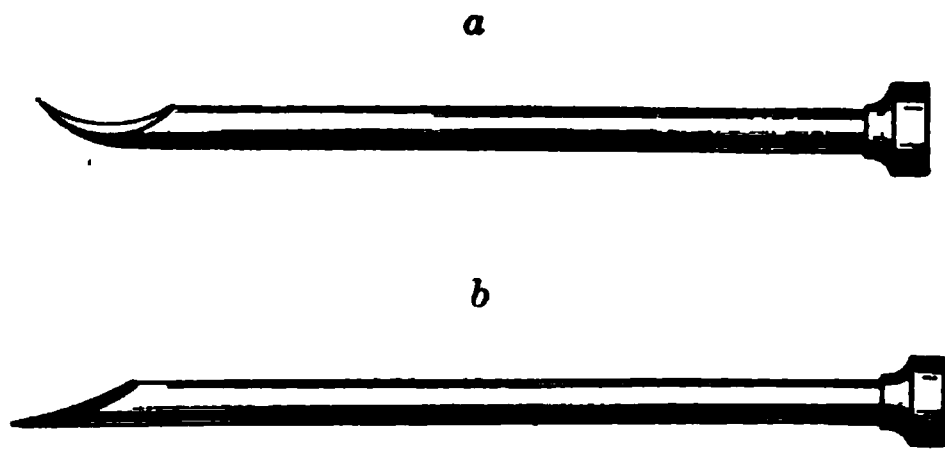
about under the point of the needle. The further away from the mouth of the vessel—that is, the nearer we approach its capillary extremity—the more favorable become the conditions for the success of the operation.

After shaving the ear and carefully washing it with clean water select the very delicate vessel lying quite close to the posterior margin of the ear, and make the injection as near to the apex of the ear as possible. At times the vessels of the ear will be found to contain so little blood that they are hardly distinguishable, making it very difficult to introduce the needle into them. This is sometimes overcome by pressure at the root of the ear, causing stasis of the blood and distention of the vessels. A very satisfactory method of causing the veins to become prominent is to press lightly or prick gently with the point of a needle the skin over the vessel to be used. In a few seconds, as a result of this irritation, the vessel will have become distended with blood, and readily distinguishable from the surrounding tissue; it may then be easily punctured by the needle of the syringe. A sharp flick with the finger will often produce the same result. The injection is always to be made from the dorsal surface of the ear.

Of no less importance than the selection of the proper vessel is the shape of the point of the needle employed. The hypodermic needles as they come from the makers are not suited at all for this operation, because of the manner in which their points are ground. If one examine carefully the point of a new hypodermic needle, it will be seen that the long point, instead of presenting a *flat*, slanting surface when viewed from the side, has a more or less *curved* surface. Now, in efforts to introduce such a needle into a vessel of very small calibre it is usually seen that the point of the

needle, instead of remaining in the vessel, as it would do were it straight (or "chisel pointed"), very commonly projects into the opposite wall; and as the needle is inserted further and further it is usually pushed through the vessel-walls into the loose tissues beyond, and the material to be injected is deposited in these tissues, instead of into the circulation. If, on the contrary, the slanting point of the needle be ground until its surface is perfectly flat when viewed from the side, and no curvature exists, then when once inserted it usually remains within the vessel, and there

FIG. 44



Hypodermic needles, magnified. *a*, improper point; *b*, proper shape of point.

is no tendency to penetrate the opposite wall. We never use a new hypodermic needle until its point is carefully ground to a perfectly flat, slanting surface with no curvature whatever.

These differences may perhaps be more easily understood if represented diagrammatically. In Fig. 44, *a*, the needle has the point usually seen when new. In Fig. 44, *b*, the point has been ground to the shape best suited for this operation. The needles need not be returned to the maker. One can grind them to the shape desired in a few minutes upon an oilstone. The size of the needle is that commonly

employed by physicians for subcutaneous injections in human beings.

When the operation is to be performed an assistant holds the animal gently but firmly in the crouching position upon a table. If the animal does not remain quiet, it is best to wrap it in a towel, so that only its head protrudes; though in most cases we have not found this necessary, particularly if the animal has not been excited prior to beginning the operation.

The ear in which the injection is to be made should be shaved clean of hair by means of a razor and soap and then washed with water. It is unnecessary to attempt disinfection of the skin.

The animal should be placed so that the prepared ear comes between the operator and the source of light. This renders visible by transmitted light not only the coarser vessels of the ear, but also their finer branches.

The filled hypodermic syringe is taken in one hand and with the other hand the ear is held firmly. The point of the needle is then inserted through the skin and into the finest part of the *ramus posterior*, the part nearest the apex of the ear, where the course of the vessel is nearly straight. When the point of the needle is in this vessel it gives to the hand a sensation quite different from that felt when it is in the midst of connective tissue. As soon as one supposes the point of the needle is in the vessel a drop or two of the fluid may be injected from the syringe, and, if his suspicions are correct, the circulation in the small ramifications and their anastomoses will rapidly alter in appearance—*i. e.*, the circulating blood will be displaced very quickly by the clear, transparent fluid that is being injected. At this stage one must proceed very carefully, for sometimes when the

needle-point is not actually in the vessel, but is in the lymph-spaces surrounding it, an appearance somewhat similar is seen. This may always be differentiated, however, by continuing the injection, when the flow of clear fluid through the vessels will not only fail to take the place of the circulating blood, but at the same time a localized swelling, due to an accumulation of the fluid injected, will appear under the skin about the point of the needle. The needle must then be withdrawn and inserted into the vessel at a point a little nearer its proximal end.

Care must be taken that no air is injected.

The hypodermic syringe and needle must, previous to operation, have been carefully sterilized in the steam sterilizer or in boiling water. The animal must be kept under close observation for about an hour after injection.

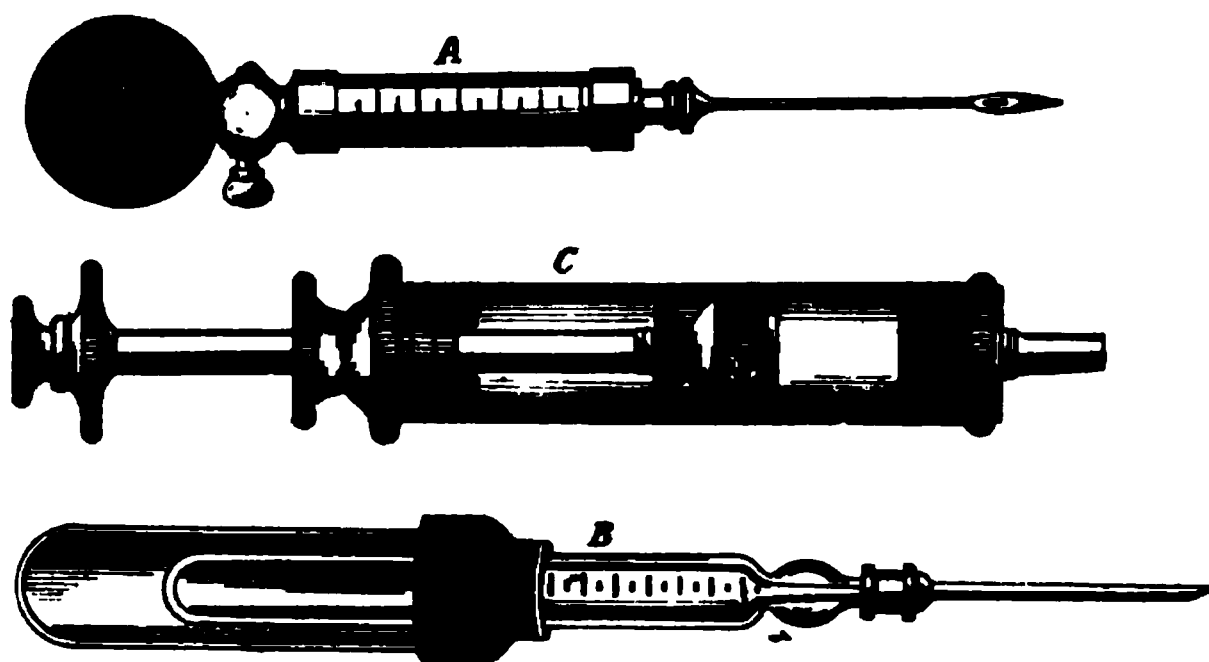
The operation is one that cannot be learned from verbal description. It can only be successfully performed after actual practice. If the precautions which have been mentioned are observed, but little difficulty in performing the operation will be experienced.

Its greater convenience and simplicity, as compared with other methods for the introduction of substances into the circulation, commend it as a technical procedure with which to make one's self familiar. The animals sustain practically no wound, they experience no suffering—at least they give no evidence of pain—and no anesthetic is required.

The form of syringe best suited for this operation is of the ordinary design, but one that permits of thorough sterilization by steam. It should be made of glass and metal, with packings that may be sterilized by steam without injury. The syringes commonly employed are those shown in Fig. 45.

For operations requiring exact dosage experience has led me to prefer a syringe after the pattern of *C*, in Fig. 45—*i. e.*, the form commonly used by physicians. The reason for this is as follows: in making injections, either into the circulation or under the skin, there is a certain amount of resistance to the passage of fluid from the needle. If one overcomes this resistance by means of a cushion of compressed air, as is the case in syringes *A* and *B*, Fig. 45, the sudden expansion of the air in the body of the syringe when

FIG. 45



Forms of hypodermic syringe. *A*, Koch's syringe; *B*, syringe of Strohschein; *C*, Overlack's form.

resistance is overcome frequently causes a larger amount of fluid to be injected than is desired. No such accident is likely to occur when the fluid is forced from the barrel of the syringe by the head of a close-fitting piston, with no air intervening between the fluid and the head of the piston. With such an instrument, properly manipulated, the dose can always be controlled with accuracy.

Inoculation into the Lymphatic Circulation.—Fluid cultures or suspensions of bacteria may be injected into the lym-

phatics by way of the testicles. The operation is in no wise complicated. One simply plunges the point of the hypodermic needle directly into the substance of the testicle and then injects the amount desired. Injections made in this manner are usually followed by instructive pathological lesions of the lymphatic apparatus of the abdomen.

Inoculation into the Great Serous Cavities.—Inoculation into the *peritoneum* presents no difficulties if fluids are to be introduced. In this case one makes, with a pair of sterilized scissors, a small nick through the skin down to the underlying fasciæ, and, taking a fold of the abdominal wall between the fingers, plunges the hypodermic needle through the opening just made directly into the peritoneal cavity. There is little or no danger of penetrating the intestines or other internal viscera if the puncture be made along the median line at about midway between the end of the sternum and the symphysis pubis. Though this may seem a rude method it is rare that the intestines are penetrated or otherwise injured. The object of the primary incision is to lessen the chances of contamination by bacteria located in the skin, some of which might adhere to the needle if it were plunged directly through the skin, and thus complicate the results.

If solid substances, bits of tissue, etc., are to be introduced into the peritoneum, it becomes necessary to conduct the operation under an anesthetic and upon the lines of a laparotomy. The hair should be shaved from a small area over the median line, after which the skin is to be thoroughly washed. A short longitudinal incision (about 2 cm. long) is then to be made in the median line through the skin and down to the fasciæ. Two subcutaneous sutures, as employed by Halsted, are then to be introduced transversely to the line of incision about 1 cm. apart, and their ends left

loose. This particular sort of suture does not pass through the skin, but, instead, the needle is introduced into the subcutaneous tissues along the edge of the incision. In this case they are to pass into the abdominal cavity and out again, entering at one side of the line of incision and leaving at the other, as indicated by the solid and dotted lines in Fig. 46. (The figure indicates the primary opening through the skin. The longitudinal dotted line shows the opening

FIG. 46

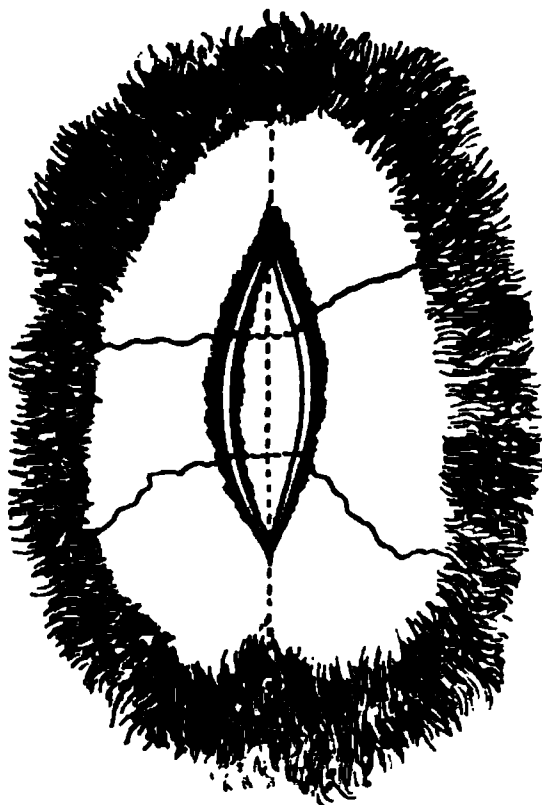


Diagram of skin incision and sutures in laparotomy on animals.

to be made into the abdomen; the transverse dotted lines, with their loose ends, represent the sutures as placed in position *before the abdomen is opened*; it will be seen that these sutures in all cases pass through the subcutaneous tissues *only* and do not penetrate the skin proper.)

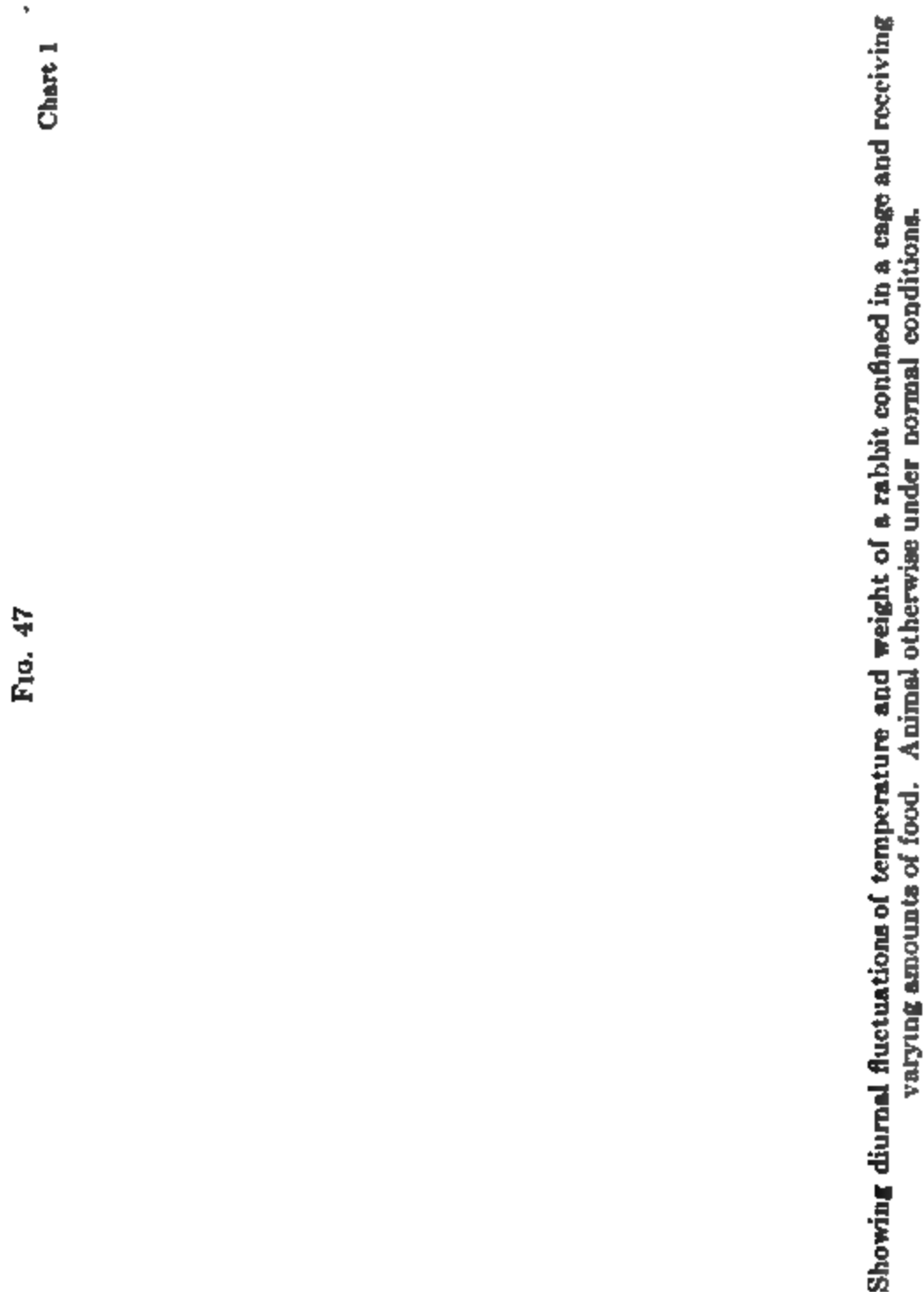
The opening through the remaining layers may now be completed; the bit of tissue is deposited in the peritoneal cavity, under precautions that will exclude all else, the

edges of the wound drawn evenly and gently together by tying the sutures, and the lines of incision dressed with collodion. It should be needless to say that this operation must be conducted under the strictest precautions, to avoid complications. All instruments, sutures, ligatures, etc., must be carefully sterilized either in the steam sterilizer for twenty minutes, or by boiling in 2 per cent. sodium carbonate solution for ten minutes; the hands of the operator, though they should not touch the wound, must be carefully cleansed, and the material to be introduced into the abdomen should be handled with only sterilized instruments.

Inoculation into the *pleural* cavity is much less frequently required—in fact, it is not a routine method. It is not easy to enter the pleural cavity with a hypodermic needle without injuring the lung, and it is rare that conditions call for the introduction of solid particles into this locality.

Inoculation into the *anterior chamber* of the eye is performed by making a puncture through the cornea just in front of its junction with the sclerotic, the knife being passed into the anterior chamber in a plane parallel to the plane of the iris. By the aid of a fine pair of forceps the bit of tissue is passed through the opening thus made and is deposited upon the iris, where it is allowed to remain, and where its pathogenic activities upon the iris can be conveniently studied. It is a mode of inoculation of very limited application, and is therefore but rarely practised. It was employed in the classical experiments of Cohnheim in demonstrating the infectious nature of tuberculous tissues, tuberculosis of the iris being the constant result of the introduction of tuberculous tissue into the anterior chamber of the eye of rabbits.

OBSERVATION OF ANIMALS AFTER INOCULATION.—After either of these methods of inoculation, particularly when

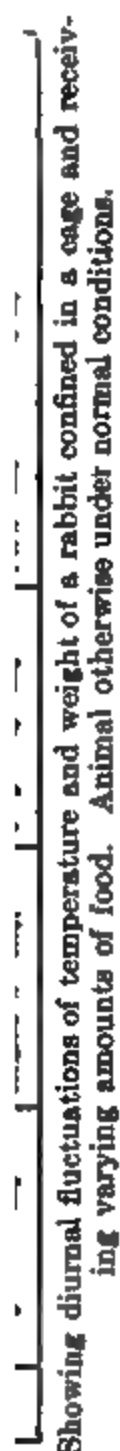


unknown species of bacteria are being tested, the animal is to be kept under constant observation and all deviations from

the normal are to be carefully noted—as, for instance, elevation of temperature; loss of weight; peculiar position in

FIG. 48

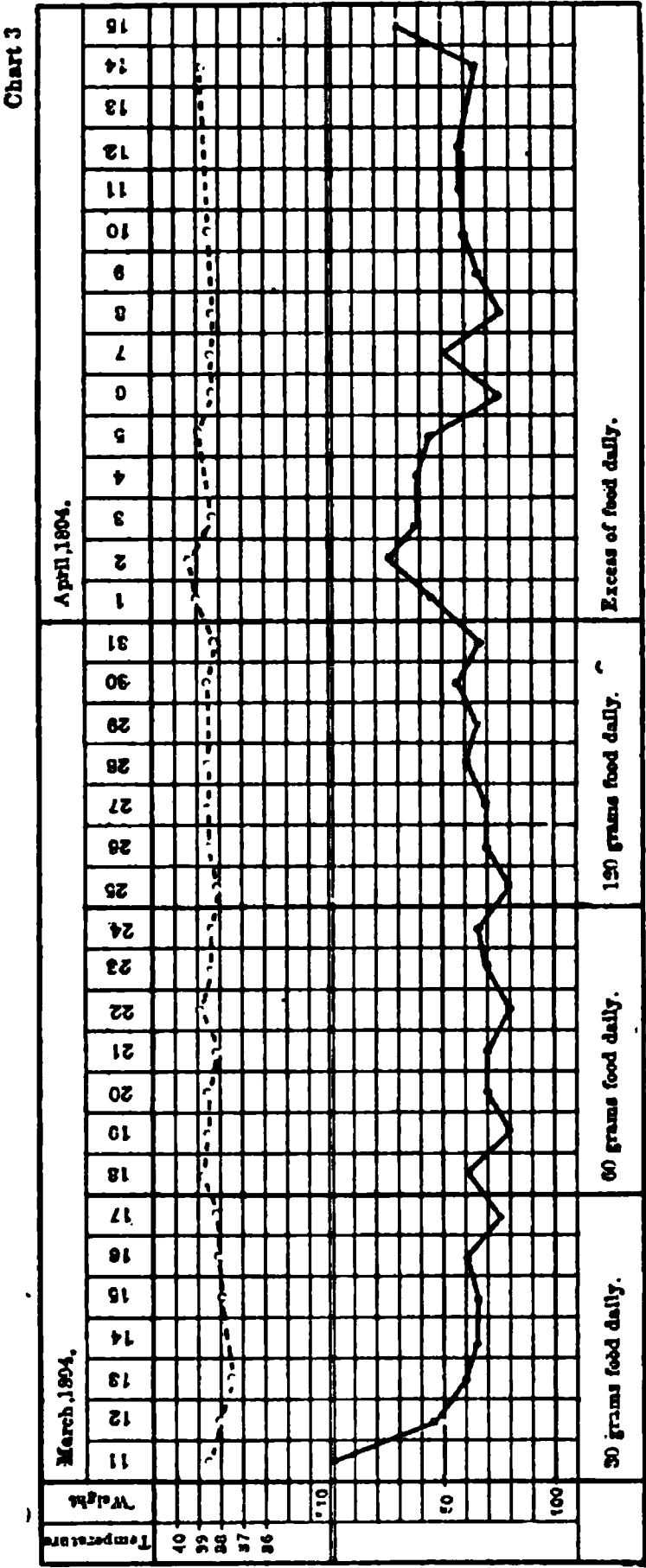
Chart 2



the cage; loss of appetite; roughening of the hair; excessive secretions, from either the air-passages, conjunctiva, or

kidneys; looseness of or hemorrhage from the bowels; tumefaction or reaction at site of inoculation, etc. If death

Fig. 49



Showing diurnal fluctuations of temperature and weight of a caged guinea-pig receiving varying amounts of food. Animal otherwise under normal conditions.

ensue in from two to four days, it may reasonably be expected that at autopsy evidence of either acute septic or toxic

processes will be found. It sometimes occurs, however, that inoculation results in the production of chronic con-

FIG. 50

Chart 4

Showing diurnal fluctuations of temperature and weight of a caged guinea-pig receiving varying amounts of food. Animal otherwise under normal conditions.

ditions, and the animal must be kept under observation often for weeks. In these cases it is important to note the

progress of the disease by its effect upon the physical condition of the animal, viz., upon the nutritive processes, as evidenced by fluctuation in weight, and upon the body-temperature. For this purpose the animal is to be weighed daily, always at about the same hour and always about midway between the hours of feeding; at the same time its temperature, as indicated by a thermometer placed in the rectum, is to be recorded.¹ By comparison of these daily observations the observer is aided in determining the course the infection is taking.

Too much stress must not, however, be laid upon moderate and sudden daily fluctuations in either temperature or weight, as it is a common observation that presumably normal animals when confined in cages and fed regularly often present very striking temporary gains and losses in weight, often amounting to 50 or 100 grams in twenty-four hours, even in animals whose total weight may not exceed 500 or 600 grams; similarly unexplainable rises and falls of temperature, often as much as a degree from one day to another, are seen. Such fluctuations have apparently no bearing upon the general condition of the animal, but are probably due to transient causes, such as overfeeding or scarcity of food, improper feeding, lack of exercise, excitement, fright, etc.

The accompanying charts (Figs. 47, 48, 49, 50) will serve to illustrate some of these points. The animals, two rabbits and two guinea-pigs, were taken at random from among stock animals and placed each in a clean cage, the kind used for animals under experiment, and kept under as good

¹ The thermometer must be inserted into the rectum beyond the grasp of the sphincter, otherwise pressure upon its bulb by contraction of this muscle may force up the mercurial column to a point higher than that resulting from the actual body-temperature.

general conditions as possible. For the first week the rabbits received each 100 grams of green food (cabbage and turnips) daily, and the guinea-pigs 30 grams each of the same food. During the second week this daily amount of food was doubled; during the third week it was quadrupled; and for the fourth and fifth weeks they each received an excess of food daily, consisting of green vegetables and grains (oats and corn). By reference to the charts sudden diurnal fluctuations in weight will be observed that do not correspond in all instances with scarcity or sufficiency of food. With the rabbits there is a gradual loss of weight with the smaller amounts of food, which losses are not totally recovered as the food is increased. With the guinea-pigs there is likewise at first a loss; but after a short time the weight remains tolerably constant, and is not so conspicuously affected by the increase in food as one might expect. From the recorded temperatures one sees the peculiar fluctuations mentioned. To just what they are due it is impossible to say. It is manifest that the normal temperature of these animals, if we can speak of a normal temperature for animals presenting such fluctuations, is about a degree or more, Centigrade, higher than that of human beings. The animals from which these charts were made were not inoculated, nor were they subjected to any operative procedures whatever, the only deviations from normal conditions being the variations in the daily amount of food given.

In certain instances, however, there will be noticed a constant tendency to diminution in weight, notwithstanding the daily fluctuations, and after a time a condition of extreme emaciation may be reached, the animal often being reduced to from 50 to 60 per cent. of its original weight. In other cases, after inoculations to which the animal is not

susceptible, rabbits in particular, if properly fed, will frequently gain steadily in weight. The condition of progressive emaciation just mentioned is conspicuously seen after intravenous inoculation of rabbits with cultures of *bacillus typhosus* and of *bacillus coli*, referred to in the chapter on the latter organism, and if looked for will doubtless be seen to follow inoculation with other organisms capable of producing chronic forms of infection, but which are frequently considered non-pathogenic because of their inability to induce acute conditions. Not infrequently in chronic infections there may be hardly any marked and constant temperature-variations until just before death, when sometimes there will be a rise and at other times a fall of temperature. In the majority of cases, however, one must be very cautious as to the amount of stress laid upon changes in weight and temperature, for unless they are progressive or continuous in one or another direction they may have little significance as indicating the existence or absence of disease.

CHAPTER XIII.

Post-mortem Examination of Animals—Bacteriological Examination of the Tissues—Disposal of Tissues and Disinfection of Instruments after the Examination—Study of Tissues and Exudates During Life.

DURING bacteriological examination of the tissues of dead animals certain precautions must be rigidly observed in order to arrive at correct conclusions.

The autopsy should be made as soon as possible after death. If delay cannot be avoided, the animal should be kept on ice until the examination can be made, otherwise decomposition sets in, and the saprophytic bacteria now present may interfere with the accuracy of results. When the autopsy is to be made the animal is first inspected externally, and all visible lesions noted. It is then to be fixed upon its back upon a board with nails or tacks. The four legs and the end of the nose, through which the tacks are driven, are to be moderately extended. Plates are now to be made from the site of inoculation, if this is subcutaneous. The surfaces of the thorax and abdomen are then to be moistened to prevent the fine hairs, dust, etc., from floating about in the air and interfering with the work. An incision is then made through the skin from the chin to the symphysis pubis. This is only a skin incision, and does not reach deeper than the fasciæ. It is best done by first making with a scalpel an incision just large enough to permit of the introduction of one blade of a blunt-pointed scissors. It is then completed with the scissors. The whole of the skin is

now to be carefully dissected away, not only from the abdomen and thorax, but from the axillary, inguinal, and cervical regions, and the fore and hind legs as well. It is then pinned flat upon the board so as to keep it as far from the abdomen and thorax as possible, for it is from the skin that the chances of contamination are greatest.

It now becomes necessary to proceed very carefully. All incisions from this time on are to be made only through surfaces that have been sterilized. The sterilization is best accomplished by the use of a broad-bladed table-knife that has been heated in a gas-flame. The blade, made quite hot, is to be held upon the region of the linea alba until the tissues of that region begin to burn; it is then held transversely to this line over about the centre of the abdomen, thus making two sterilized tracks, through which the abdomen may be opened by a crucial incision. The sterilization thus accomplished is, of course, directed only against organisms that may have fallen upon the surface from without, and therefore, it need not extend deep down through the tissues. In the same way two burned lines may be made from either extremity of the transverse line up to the top of the thorax.

With hot scissors the central longitudinal incision extending from the point of the sternum to the genitalia is to be made without touching the internal viscera. The abdominal wall must therefore be held up during the operation with sterilized forceps or hooks. The cross-incision is made in the same way. When this is completed an incision through the ribs with a pair of heavy, sterilized scissors is made along the scorched tracks on either side of the thorax. After this the whole anterior wall of the thorax may easily be lifted up, and by severing the connections with the diaphragm it may be completely removed. When this is done

and the abdominal flaps laid back, the contents of both cavities are to be inspected and their condition noted without disturbing them.

After this the first steps to be taken are to prepare plates or Esmarch tubes from the blood, liver, spleen, kidneys, and any exudates that may exist. This is best done as follows: Heat a scalpel quite hot and apply it to a small surface of the organ from which cultures are to be made. Hold it upon the organ until the surface directly beneath is visibly scorched. Then remove it, heat it again, and while quite hot insert its point through the capsule of the organ. Into the opening thus made insert a sterilized platinum loop, made of wire a little heavier than that

FIG. 51



Nuttall's platinum spear for use at autopsies.

commonly employed. Project this deeply into the tissues of the organ; by twisting it about enough material from the centre of the organ can be obtained for making the cultures.

As the resistance offered by the tissue is sometimes too great to permit of puncture with the ordinary wire loop, Nuttall¹ devised for the purpose a platinum-wire spear which possesses great advantages over the loop. It has the form seen in Fig. 51. It is easily made by beating a piece of heavy platinum wire into a spear-head at one end, and perforating this with a small drill, as seen in the cut. It is attached by the other end to either a metal or glass

¹ Centralblatt für Bakteriologie und Parasitenkunde, 1892, Bd. xi, p. 538.

handle, preferably the former. It can readily be thrust into the densest of the soft tissues, and by twisting it about after its introduction particles of the tissue sufficient for examination are withdrawn in the eye of the spear-head.

Cultures from the blood are usually made from one of the cavities of the heart, which is always punctured at a point which has been burned in the way given.

In addition to cultures, cover-slips from the site of inoculation, from each organ, and from any exudates that may be present must be made. These, however, are prepared *after* the materials for the cultures have been obtained. They need not be examined immediately, but may be placed aside, under cover, on bits of paper upon which the name of the organ from which they were prepared is written.

When the autopsy is complete and the gross appearances have been carefully noted, small portions of each organ are to be preserved in 95 per cent. alcohol for subsequent examination. Throughout the entire autopsy it must be borne in mind that all cultures, cover-slips, and tissues must be carefully labelled, not only with the name of the organ from which they originate, but with the date, designation of the animal, etc., so that an account of their condition after closer study may be subsequently inserted in the protocol.

The cover-slips are now to be stained, mounted, and examined microscopically, and the results carefully noted.

The same care with regard to noting, labelling, etc., should be exercised in the subsequent study of the cultures and the hardened tissues, which are to be stained and subjected to microscopic examination. The results of microscopic study of the cover-slip preparations and of those obtained by cultures should in most cases correspond,

though it not rarely occurs that bacteria are present in such small numbers in the tissues that their presence may be overlooked microscopically, and still they appear in the cultures.

If the autopsy has been performed in the proper way, with the precautions given, and sufficiently soon after death, the results of the bacteriological examination should be either negative or the organisms which are isolated should be in pure cultures. This is particularly the case with cultures made from the internal viscera.

Both the cover-slips and cultures made from the point of inoculation are apt to contain a variety of organisms.

If the organism obtained in pure culture from the internal viscera, or those predominating at the point of inoculation of the animal, have caused its death, then subsequent inoculation of pure cultures of this organism into the tissues of a second animal should produce similar results.

When the autopsy is quite finished the remains of the animal should be *burned*; all instruments subjected to either sterilization by steam or boiling for fifteen minutes in a 1 to 2 per cent. soda solution; and the board upon which the animal was tacked, as well as the tacks, towels, dishes, and all other implements used at the autopsy, be sterilized by steam. All cultures, cover-slips, and, indeed, all articles likely to have infectious material upon them, must be sterilized as soon as they are of no further service.

What has been said with regard to the study of dead tissues obtained at autopsy applies equally well to the bacteriological study of tissues and exudates obtained during life. In the latter case, however, certain precautions are always to be observed. In the first place, it is desirable to

obtain the materials under aseptic precautions, care being taken that no disinfectant fluids are mixed with them. They should be subjected to study as soon as possible after removal from the body. In the case of tissues that cannot be examined on the spot, they should be placed in a sterile Petri dish or in a stoppered, sterile, wide-mouthed bottle and taken at once to the laboratory. The surface should then be seared with a hot knife and an incision through the seared area into the centre made with a knife that has been sterilized and allowed to cool. From the depths of this incision enough material may be obtained for microscopic examination and for the preparation of cultures. Fluid exudates that must be taken to the laboratory should be collected in either a sterile test-tube, or, better, in a sterile capillary tube that is subsequently sealed at both ends in a gas-flame. When bacteriological examination of the blood during life is required, it is customary to obtain the necessary sample of blood by pricking the skin. It must be remembered, in this connection, that the skin usually contains a number of species of bacteria that are of no pathological significance and have nothing to do with the disease from which the individual may be suffering. It is manifestly essential to exclude these. It is not possible to exclude them certainly and completely under all circumstances, without a more or less elaborate procedure; but an effort to do so should always be made. As a rule, the greater number of them may be removed from the skin by careful washing with warm water and soap and a sterile brush, after which the skin should be rinsed with alcohol and allowed to dry spontaneously. The drop of blood may then be obtained from the skin thus cleaned by a prick with a sharp, sterilized lancet. The presence in the cultures

of a staphylococcus, growing slowly, with white colonies, is a frequent experience, and does not necessarily imply that this organism bears an etiological relation to the disease from which the individual may be suffering (see *Staphylococcus Epidermis Albus*).

When more than a few drops of blood are needed, as may be the case in deciding the general nature of an infection process, it is customary to withdraw it from one of the superficial veins of the forearm by means of an hypodermic syringe. The operation should be done under strictly aseptic conditions, *i. e.*, the skin should be thoroughly cleaned with soap, water, and alcohol; the hands of the operator should be surgically clean; the syringe must have been sterilized immediately before using, and great care should be taken that no air bubbles be injected into the veins during the operation.

In interpreting the results of cultures made from blood drawn in this manner, the possibility of contamination by skin bacteria should not be forgotten. The success of the operation depends upon attention to the most minute details of aseptic practice. It requires for its safe practice skill in manipulation, experience and judgment in the interpretation of the results. It is not, therefore, an operation to be commended to the beginner.

"ULTRA"-MICROSCOPIC OR "FILTERABLE" VIRUSES.

These terms relate to particular substances capable of causing disease, that are so small as to be beyond the visual range of the microscopes used in bacteriological work and to be able, because of their minute dimensions, to pass through the pores of the finer grades of earthenware filters.

Their existence has been suspected for a number of years but it is only comparatively recently that sufficient became known of them to justify our speaking confidently of them; and even now little more than their etiological potentialities and some of their physiological reactions can be considered.

For a long while it has been a puzzle that such characteristic contagious diseases as certain of the acute exanthemata in man and a number of typical transmissible diseases in animals should have eluded all efforts to discover their causes. By the customary methods of bacteriological analysis nothing of a positive character is learned and yet by the introduction into susceptible animals of bits of tissue from the diseased animal, or small quantities of blood or tissue juices or even of filtrates of emulsions of such tissues or juices, it is possible in a number of instances to reproduce the disease. It is such evidence as this that serves as the basis for the belief in the existence of invisible viruses for a number of diseases of man and animals and a few for plants.

The existence of such viruses has been demonstrated in smallpox vaccine, yellow fever, measles, typhoid fever, dengue fever, poliomyelitis, and trachoma, among the diseases of man and in foot and mouth disease, contagious pleuropneumonia, sheep-pox, rabies, cattle plague, chicken sarcoma, and distemper of dogs among those of animals, and in the mosaic disease of the tobacco plant.

Though little or nothing that is convincing can be said of the morphology of this group of ultra-microscopic particles, still in their reactions to a variety of physical agents they are obviously living matter, having many analogies to the more highly developed microorganisms with which we are familiar. Practically all are killed at temperatures

ranging from 55° to 70° C. Some resist drying for comparatively long periods of time, others are quickly killed by it. Practically all are resistant to the action of glycerin. This is not the case as a rule with bacteria. They vary considerably in their resistance to such germicidal substances as formalin, boric acid, corrosive sublimate and menthol.

Practically all animals that survive their invasion have acquired immunity from a second attack of the disease. There is little evidence that the growth is accompanied by the production of toxins as such. A survey of such data as are available justifies the suspicion that these bodies are more closely allied to the protozoa than to the bacteria.

Efforts at cultivation under artificial circumstances have succeeded in only a few instances. In their studies upon the contagious pleuro-pneumonia of cattle Nocard and Roux by the use of special methods, both optical and cultural, claim to have demonstrated the causative factor of that disease. The method employed by them for the cultivation of the virus is that suggested by Metchnikoff,* Roux and Salambini in 1896. It consists in placing bits of tissue or secretions from the infected animals in small, sterilized collodion sacs, which are finally hermetically sealed with sterile collodion. These little sacs with their contents are then placed in the peritoneal cavity of an animal; a rabbit, chicken, guinea-pig, calf, dog, or sheep as the case may be, and left there for a time. The idea on which this method is based is that the collodion sacs are impermeable for the specific virus but are permeable to the normal juices of the peritoneal cavity of the animal in which they are placed. Under these circumstances the specific virus was expected to develop within the sacs and receive its food supply by diffusion from the surrounding peritoneum; the body tem-

perature of the animal in which they were placed being most favorable to incubation.

The investigators found that by the use of a special system of illumination and very high magnification, about 2000 diameters, there were to be detected within the collodion sacs, in from a few days to several weeks, numerous motile points or dots of such minute dimensions that it was often impossible to decide as to their exact form. No such bodies were seen in control collodion sacs placed similarly in the peritoneum of animals but in which sacs none of the tissue or juices from a diseased animal had been inclosed. Nocard and Roux are disposed to regard these bodies as the exciting cause of the disease under consideration.

Flexner and Noguchi announce that by the use of Noguchi's method for cultivating spirochetæ they have isolated from the central nervous tissues of both man and monkeys dead of poliomyelitis, minute coccus-like bodies that they believe to be the cause of the disease. The culture medium consists of human ascetic fluid to which a fragment of sterile fresh rabbit kidney has been added. The cultivation is conducted at first under anaërobic conditions but later subcultures do not demand complete absence of free oxygen. When ready the tubes are inoculated with small bits of the diseased cerebrum or cord after which a thick layer of sterile paraffin oil is placed upon the surface of the ascetic fluid.

This suffices for the exclusion of free oxygen. After from seven to twelve days at body temperature a diffuse clouding or opalescence appears about the bit of nervous tissue in the tube. Microscopic examination of this opalescent matter reveals the presence of coccoid bodies conspicuous for their variation in size.

Their true nature has not been determined. The disease

can be reproduced in monkeys by inoculation with the cultures, but not with regularity.¹

By an analogous method Noguchi has cultivated from both rabies and trachoma bodies that he regards as etiologically related to the diseases from which they were obtained. It is not possible as yet to be either certain as to the accuracy of his suspicions or to satisfactorily classify the bodies found in his cultures. In some respects they suggest bacteria, in some protozoa and taking them in conjunction with the tissue findings in the diseases it seems fair to suspect that they may be developmental forms of the Negri bodies constantly present in rabies in the one case or the singular cell inclusions common to trachoma, the so-called "trachoma bodies" in the other.²

In the study of many of the common diseases, notably the exanthemata, both at autopsy and during life, by the methods above outlined, the investigation often yields negative results, and yet there is every reason for believing these diseases to be dependent for their existence upon invasion of the body by some form or another of living micro-organisms, capable of growth in the tissues and susceptible of being transmitted from individual to individual, either directly or indirectly. It is possible that the application of one or another of the foregoing methods to the study of these diseases may demonstrate that some of them at least are due to the presence of so-called filterable viruses.

¹ For details see Flexner and Noguchi, *Jour. Exp. Med.*, 1913, vol. xviii, No. 4.

² For particulars see Noguchi, *Jour. Exp. Med.*, 1913, No. 4; *ibid.*, 1913, No. 5.

CHAPTER XIV.

Infection and Immunity—Mechanism—Specific Bodies and Reactions—
Doctrines in Explanation.

INFECTION.

IF one examines in detail the lesions resulting from the invasion of the body by the different types of infective bacteria, justification is found for the conclusion that the physical manifestations of infection, that is, the sites of activity and the characteristic lesions, vary with the nature of the different invading parasites.

To a certain extent this is true; that is to say, the type of lesion characterizing a specific disease is peculiar to that disease and is produced only by the particular micro-organism having the power to excite the disease. But if we take up the various lesions of specific diseases in intimate detail we shall see, as will be shown later, that fundamentally the essential factor in the mechanism of infection is of the same general nature for all diseases, be the characteristic lesions and clinical manifestations what they may; the apparent differences being referable to dissimilarities of structure and function of the various species of bacteria that excite the several phenomena on the one hand, and to the parts of the body of the host that are attacked on the other. Thus, by way of illustration, if we select a group of clinically and pathologically distinct infections, such as anthrax, miliary tuberculosis, and diphtheria, and compare the conditions recorded at autopsy, little of a macroscopic nature will be

discovered to suggest anything that is common to all, and even if the tissues be examined microscopically such marked divergencies are seen that we are still in doubt as to the existence of a common factor. In the case of anthrax, a true septicemia, the blood current is the seat of activity of the exciting bacteria, and beyond congestion, enormous numbers of bacteria in the bloodvessels and the escape of serum into the tissues (edema), little else is to be seen to account for death. On the other hand, in the case of miliary tuberculosis, even though the involvement of the organs may be general, there is no similar invasion of the blood stream. The tubercles are circumscribed, are often surrounded by healthy tissue and, though obviously distributed throughout the body from a primary focus through the agency of the circulating fluids, each tubercle may nevertheless be regarded as a distinct local infection. There is, however, a conspicuous difference between the lesions found here and those seen in anthrax. The lesion of tuberculosis, the tubercle, is always characterized by tissue death at and about its centre, *i. e.*, where the bacilli are located, even in the earliest stages of its development.

On postmortem examination of an animal dead of diphtheria we observe conditions that are unlike those noted in both anthrax and tuberculosis. There is neither an invasion of the vascular system nor a distribution of conspicuous pathological foci throughout the body. The bacteria are confined to the primary site of invasion and when found in distal organs are there only in small numbers and give no evidence of an effect upon the tissues immediately surrounding them.

Thus far, as a result of this review, we have two points in common to the three distinct diseases, viz.: they are all

caused by bacteria, and they all may terminate fatally. On the other hand the clinical symptoms and the pathological lesions are such as to characterize each as a pathological entity. But, as has been intimated, there is a fundamental factor common to all, and the discovery of this factor gives the clue to the true mechanism of all infections. Light upon this phase of the subject can best be secured through experimental methods.

Observation and experiment have taught us that sometimes highly pathogenic bacteria may lose in part or in whole their disease producing properties without at the same time losing their vitality. If such "attenuated" bacteria be injected into susceptible animals the result may be nothing; or it may be a modified lesion totally dissimilar to that following injection of the fully virulent organism. This is often the case with the bacteria that excite septicemia, and the bacillus causing anthrax serves as a useful illustration. When normal, as it is usual to regard it, it is fully virulent and causes fatal blood poisoning in susceptible animals, but if subjected to certain chemical or physical influences the virulence may gradually be lessened until finally we may have a living anthrax bacillus that has been deprived of almost all its disease producing power. If animals be inoculated with such attenuated anthrax bacilli the conditions found may be in striking contrast to those produced by the normal germ. Instead of the bloodvessels being almost packed with bacteria, they may contain few or none, and the only bacteria to be found in the body in numbers are at and immediately about their point of deposit. Yet these animals exhibit clinical symptoms and occasionally die.

Similarly, in other varieties of septicemia, the so-called

"hemorrhagic group" we see as a rule typical, fatal septicemias resulting from the invasion of the body by the organisms causing them; but at times, through influences not fully known, these organisms become modified in their physiological functions so that instead of the customary general invasion of the circulating fluids there may be only a very slight invasion and the results of their inoculation are principally evidenced as local destruction of tissue, sometimes with fatal results. Obviously then these organisms have the power of causing constitutional disturbances, tissue changes and even fatal results without the necessity of their being themselves disseminated throughout the body by way of the circulating fluids.

As said above the characteristic lesion of tuberculosis is the tubercle, and the peculiarity of the tubercle is necrosis, observable almost from the moment it begins to develop. If tuberculosis be induced through the intravenous injection of rabbits with carefully prepared suspensions of living virulent tubercle bacilli the resulting miliary tubercles are always marked by more or less death of tissue at and about their centre, which tissue death progresses as the disease progresses, until it reaches a point easily seen with the naked eye and finally incompatible with life. If on the other hand a similar injection be made with a suspension of tubercle bacilli that have been killed, by heat or otherwise, disseminated nodules, tubercles, will also be found in the internal organs. These may be, histologically, strikingly like those following the use of the living organism; they are marked by the characteristic tissue death, but it is less in evidence and it is not progressive beyond certain limits and the injection does not necessarily prove fatal to the animal. As a result of this experiment we see that dead bacteria may produce a result differing

only in degree from that caused by the same species when living and fully virulent.

A similar property may be demonstrated in a number of other pathogenic species in no way related to *Bacillus tuberculosis*. Obviously, there is something within or associated with these bacteria that may act upon the tissues even though the bacteria themselves may be dead.

In our autopsy on the animal dead of diphtheria we saw that the bacilli were not distributed throughout the body, but were confined to the site of inoculation. We saw at the site of inoculation a tissue reaction scarcely sufficient to account for the fatal result, yet that result occurred within a comparatively short time after inoculation.

When diphtheria occurs in human beings the same holds true as a rule, and while occasionally the local reaction in the throat is such as gravely to imperil life through obstruction to respiration, the real danger in most cases is not local but remote, and the clinical observations on the living subject affected with this disease point to the far-reaching influence of a local phenomenon, that, of itself, may often seem to be of but slight significance.

If the internal organs of either animals or human beings that have died of diphtheria be examined microscopically, changes are easily to be discovered that are incompatible with life and that at once account for many of the clinical manifestations of the disease, yet these changes are not accompanied by the presence of bacteria nor by any other agent that can be detected by the eye.

It is plain, then, that the serious influence of the local infection of diphtheria is referable to a something that originates at the point where the bacteria are growing and is from that point distributed to the distant organs.

Has the specific germ of diphtheria any property to warrant such a view? If a fluid culture of *bacillus diphtheriæ* be filtered through a porcelain filter, the filtrate will contain none of the bacteria. If this filtrate, free of all bacteria, be injected into animals, death ensues; and if the tissues of these animals be examined, all of the most important lesions that characterized the tissues of the animal dead after inoculation with the living germ are to be found.

If a parallel experiment be made with the *bacillus* of tetanus analogous results will be obtained.

It is clear, then, that here are two species of bacteria that excite the characteristic results through the instrumentality of a something that they manufacture in the course of their growth; that may be separated from them by the simple process of filtration, and that when so separated possesses all the properties of specific intoxicants.

In anthrax and other septicemias we saw that, normally, the infection was characterized by the distribution of the bacteria throughout the body, but that modified results, differing only in degree, might still be obtained with the attenuated organisms without such general distribution. These latter conditions must, therefore, have been caused by a poison elaborated by or escaping from the locally deposited organisms and carried to distant parts of the body by the circulating fluids. In tuberculosis the nodules resulting from inoculation with the dead bacteria must have been the result of a poison associated with the bodies of those dead bacteria and liberated with their disintegration in the tissues; while in diphtheria it is plain that its characteristic manifestations are the outcome of a poison produced locally by the growing bacteria and carried thence by the circulating fluids to distant organs, there to exhibit its destructive properties.

Thus far, then, infection must be viewed as a conflict between bacteria on the one hand and tissues on the other; the former having as their weapons of offence destructive poisons; the latter, vital defensive provisions that enable them to resist infection with greater or less degree of success, according to circumstances. It makes no difference, therefore, whether, in infection, the bacteria be generally or only locally present, the mechanism of infection is at bottom a destructive intoxication.

Bacterial Toxins.—The term “toxins,” as used in bacteriology, refers to a group of soluble, nitrogenous, non-crystallizable poisons that are elaborated by certain bacteria in the course of their growth, both in the tissues of the living host and under conditions of artificial cultivation. They are assumed to be by-products of metabolism and they may be separated easily from the living bacteria by which they are manufactured by the simple process of filtration through fine-pore earthenware filters. As they have not been obtained in a pure state their chemical composition cannot be stated precisely but it is probable that they are allied to the globulins, nucleo-albumens, peptones, albumoses, or the enzymes.

The toxins are identified, not by their chemical structure, but rather by their harmful action upon the tissues of living animals, *i. e.*, by their physiological reactions. It is this property that renders them of such significance in the phenomena designated as disease.

By the injection of either of these bacteria-free, true toxins into the tissues of susceptible animals, lesions are produced that are in all essential respects identical with those occurring in the course of infection by the living bacteria. By varying the dose of toxin injected into the animal

one may produce either prompt death or only slight constitutional reaction. In the latter event repeating the injection of a non-fatal dose may have no apparent effect upon the animal. In such a case the animal has acquired, loosely speaking, a tolerance to the poison and this tolerance is due to a newly formed, antidotal substance now circulating in the blood of the tolerant or immune animal. For example: If a measured quantity of the toxin under consideration be mixed in test-tubes with varying amounts of the serum of the tolerant animal and each of these mixtures be injected into fresh, normal animals of the same species, it will be seen that in some instances the toxicity of the poison is only lessened, while in others it may be completely neutralized; in other words, we have demonstrated by such an experiment the presence in the blood of an antidote, and "antitoxin" as it is called. This antidote is specific, that is, it can neutralize only the poison used in the experiment; it is inactive when used against other toxins.

This union between toxin and its antidote is conceived to occur according to the laws governing ordinary chemical reactions, *i. e.*, there is a definite numerical relationship; a certain fixed quantity of toxin being neutralized by a certain fixed amount of antitoxin, variations in either factor resulting in failure to accurately neutralize. The union between the two factors is made possible, according to Ehrlich's conception, through the possession by the toxin molecule and by the antitoxin molecule of constituents having the combining function, "haptophore" side chains, as he calls them. In addition the toxin molecule possesses another constituent having the poisonous destructive function, the "toxiphoric," side chains, while the antidotal or antitoxic molecule possesses a constituent having the neu-

tralizing function. Of the functions of these side chains, that of combination is the more permanent.

Toxoids and Toxones.—Bearing this matter of permanency in mind we find that when toxins are allowed to stand, acted upon by heat, light and air, for a time, they may still combine, as may be determined numerically, with the appropriate antidotes or antitoxins, but may show evidence of diminution of their intoxicating principle. When in this degenerated state they are designated as “toxoids” and “toxones.”

A point of peculiar interest in connection with the true bacterial toxins is the extraordinary toxicity of those with which we are more or less fully acquainted. Experiment leads to the belief that the toxins of diphtheria and of tetanus are more highly poisonous than any other known poisons. Thus, for instance, diphtheria toxin is capable of causing fatal intoxication in a guinea-pig weighing 400 grams when injected subcutaneously in so small a dose as 0.05 milligram,¹ while typical tetanus is produced in a mouse by the injection of 0.0001 milligram of tetanus toxin.²

The number of bacteria capable of elaborating true toxins is very small; indeed, in so far as those of significance to animal pathology is concerned, we are certain of only two species having this property, viz., the bacillus of diphtheria and the bacillus of tetanus. For most of the other pathogenic species their toxic action is referable, not to toxins, as defined above, but rather to toxic components of the bacterial cells, the endotoxins or intracellular toxins.

The Endotoxins or Intracellular Toxins.—The term Endotoxin is generically used to designate a toxic, protein component of the bacterial cells, *i. e.*, it is part and parcel of the

¹ Roux and Yersin, *Annals de l'Inst. Pasteur*, 1889, iii, p. 287.

² Brieger and Cohn, *Zeit. f. Hyg. u. Infekt.*, 1893, Bd. xv, Heft 1.

cell and becomes active, presumably, only when the cells are disintegrated. Such disintegration may occur as a result of autolysis or self-digestion of the bacteria under special conditions of artificial cultivation, or it may be seen as the outcome of the lytic or solvent action of the resisting body cells or fluids, either those of the infected animal or, as in the case of the toxins and antitoxins, those of the animal that has become tolerant in one way or another to the activities of the bacteria in question. Endotoxins are not liberated from the bacterial cells as a secretion or excretion or manufactured as an extracellular by-product, as is the case of the toxins, but are peculiarities of the protoplasm of which the bacteria are composed.

The escape of endotoxin from bacterial cells as a result of autolysis is seen occasionally in old cultures that have been kept for a time under more or less constant conditions. It is probable that it occurs to a limited degree in all cultures of endotoxic bacteria as a result of the death and final dissolution of a smaller or larger number of individual bacteria in such cultures. For want of a better interpretation this liberation is supposed to be the result of a sort of self-digestion by enzymes that are within the bacteria as normal components. It is most conspicuously to be seen in cultures of those endotoxic species that most readily undergo those morphological changes commonly denominated as involution or degeneration; the spirillum of Asiatic cholera and the meningococcus may be cited as conspicuous illustrations. The fundamental mechanisms of this phenomenon cannot be discussed with profit as little or nothing is known of it.

As in the case of toxins, the definite chemical nature of endotoxins cannot be stated. Nevertheless Buchner isolated

from a number of bacterial species protein constituents, "bacterio proteins," as he denominated them, having the common properties of solubility in alkalies, relative resistance to the boiling temperature, attraction for leukocytes (positive chemotaxis), and pyogenic powers.

The liberation of endotoxins from the bacterial cells by strictly bacteriolytic processes going on in the living body is not a simple phenomenon. It is conceived as resulting from a solution of the bacteria by a certain ferment-like body in the blood. This ferment-like body can act only when it is bound to the bacterial cell by a specific intermediary body. This latter is supposed to be portions of cells that have been thrown off from fixed cells in the course of immunization, *i. e.*, in the course of acquiring tolerance to the action of certain endotoxic bacteria.

In numerous instances bacteria are disintegrated by normal blood. Here it is believed that the ferment-like body is brought into action through the agency of intermediary bodies of a non-specific nature, *i. e.*, of bodies normally present that may have the power to bind any or all bacteria to the ferment-like body and thus lead to their destruction.

While the blood of all animals possesses some destructive solvent or disintegrating action for most bacteria, this is never so great as is that of the blood of immunized animals upon the particular bacteria from which they are immune.

Endotoxins Distinct in their Action from Toxins.—Like toxins, endotoxins, *i. e.*, dead endotoxic bacteria, may cause disease and death of the animal tissues. Similarly, when endotoxic bacteria are repeatedly injected in sublethal doses, immunity of varying degrees develops. The immunity resulting from the use of non-fatal doses of endotoxic bacteria, is not, however, an antitoxic or an anti-endotoxic

immunity, but the substance appearing in the blood of animals so immunized is rather bacteriolytic, and the blood of such animals may contain little or no true antitoxic components.

Moreover, if the blood serum of an animal immune from true toxin be injected into a normal animal, this latter at once acquires some degree of resistance to the toxin from which the first animal was protected, *i. e.*, it is "passively" immunized; on the other hand, if the bacteriolytic serum of an animal artificially immunized from endotoxic bacteria be similarly transferred to a normal animal, there is no similar transference of the state of immunity.

THE DEFENSES OF THE BODY.

When considered in the most comprehensive way we find that the normal body is endowed with a number of natural provisions that may fairly be regarded as defenses against the invasion of hurtful parasites. Thus for instance: If the skin of even the most cleanly persons be examined bacteriologically, we find that in the majority of cases bacteria of several kinds, often those having the power to cause disease, are to be detected. So long as the skin is intact and the individual in good general health no harm results. The reason for this is found in the structure of the skin. The horny epidermis and the fat and sweat secretions serve as effectual barriers against both the multiplication of germs and their penetration into the underlying tissues. The hairs about the orifices act to some extent as filters or screens for bacteria laden dust; the ciliated epithelium of the upper air passages serves as a sweep to rid the body of foreign particles that may find lodgment upon it; and the acid reaction

of the gastric juice, low though it be, is thought sufficient to render inert certain infective bacteria that enter the alimentary tract by way of the mouth. Of all the defenses, however, none are certainly of so much importance as those to be detected within the internal structures of the normal animal. In its warfare against the invasion of infective bacteria and the activities of their poisonous products, the most significant defenses possessed by the body are those which directly aim at the destruction of the living germs of disease and at the neutralization of their poisonous waste products.

In so far as we now know the internal means of defense used by the body in its warfare against infective bacteria and their poisonous products are the phagocytic cells, such as the leukocytes, the large mononuclear cells of the blood, and the connective tissue and endothelial cells, and the ill-defined vital substances in the circulating blood which act, so to speak, as antidotes to bacterial poisons. If these defenses are not of sufficient vigor to destroy the invading bacteria, or to render inert the poisons produced by them, the bacteria are victorious and infection results; on the other hand, if there be failure to excite disease, the tissues have been victorious, and are then said to be *resistant* to or *immune* from this or that particular type of infection.

In some cases the protective agents possessed by the animal organism act directly upon the invading parasites themselves—*i. e.*, they are germicidal; in others their function is more that of antidotes, or neutralizers in the chemical sense, of the poisons produced by these parasites, the parasites themselves, in certain instances, experiencing only slight injury from a limited sojourn in the living tissues.

So far as we can learn the blood serum exhibits normally

a small amount of antitoxic, agglutinative, and bactericidal action against a great variety of pathogenic bacteria. The nature of the agents responsible for these activities is believed to be identical with that of similar agents found in the blood of artificially immunized animals, though in the latter instance they are always present to a higher degree than in normal animals.

To those ill-defined substances whose affinities are restricted to the soluble toxins elaborated by the invading bacteria the name "antitoxins" is now generally applied. Contrary to what we have seen in the case of the germicidal substances, normally present in the blood, antitoxins are to be detected in the normal animal organism in very small amounts. When they do exist under such conditions they are of but comparatively feeble potency.¹

In the great majority of instances antitoxic activities are acquired peculiarities; acquired in some cases in a more or less natural manner, as in the course of a non-fatal attack of a specific malady; induced in others by purely artificial means, as in the case of immunization from diphtheria and tetanus.

Our acquaintance with the antitoxins extends little beyond their physiological functions and some of the means that induce their generation. We have no satisfactory knowledge of their intimate nature or of the primary sources of their production. They are believed by some (Buchner² and Metchnikoff³) to represent, when artificially induced, bac-

¹ See Bolton, Transactions of Association of American Physicians, 1896, xi, 62. Pfeiffer, Deutsche med. Wochenschrift, 1896, No. 8. Fischl and v. Wanschheim, Centralblatt für Bakteriologie, Parasitenkunde, und Infektionskrankheiten, 1896, Abt. i, Bd. xix, S. 652. Wassermann, Berliner klin. Wochenschrift, 1898, No. 1.

² Münchener med. Wochenschrift, 1893, Nos. 24 and 25.

³ Weil's Handbuch der Hygiene, Bd. ix, Lieferung 1, S. 48.

terial toxins that have been modified by the vital action of the integral cells of the body; and Roux¹ and Buchner² maintain that they exhibit their protective functions less by direct combination with the toxins than by a specific stimulation of the tissue-cells that enables the latter to resist the harmful influences of the toxins. On the other hand, Behring,³ Ehrlich,⁴ and their associates contend that they are vital tissue elements, having the property of combining directly with the toxins to form "physiologically inert toxin-antitoxin" compounds that are in a manner analogous to the double salts of familiar chemical reactions.

Natural Immunity.—It is well known that among man and the lower animals individuals are frequently encountered who are, in general, less susceptible to infection than are others of their species; and that particular species of animals not only do not suffer naturally from certain specific diseases, but resist all efforts to produce the diseases in them by artificial methods; in other words, they are *naturally immune* from them. The term "natural immunity," as here employed, implies a congenital condition of the individual or species, a condition peculiar to his idioplasm, which has been transmitted to him as a tissue-characteristic through generations of progenitors.

Acquired Immunity.—Again, it is often observed that an individual or an animal after having recovered from certain forms of infection has thereby acquired protection from subsequent attacks of like character; in other words, they are said to have *acquired immunity* from this disease. "Ac-

¹ Annales de l'Institut Pasteur, 1894, p. 722.

² Berliner klin. Wochenschrift, 1894, No. 4.

³ Infektion und Disinfektion, Leipzig, 1894, S. 248.

⁴ Klinisches Jahrbuch, 1897, Bd. vi, Heft 2, S. 311. Fortschritte der Medicin, 1897, Bd. xv, No. 2.

quired immunity" implies, therefore, a condition of the tissues of an individual, not of necessity peculiar to other members of the race or species, that has originated during his life from the stimulation of his integral cells by one or another of the specific infective irritants that may have been purposely introduced, or accidentally gained access to his body. Acquired immunity may be either active or passive in character.

Active Immunity.—Active immunity is that seen after recovery from infection acquired in a natural way, or from infection induced by the injection of dead or living organisms or the poisons peculiar to them.

Passive Immunity.—Passive immunity is that condition in which protective substances that have been generated in a susceptible animal by one or the other methods of active immunization are transferred directly from that animal to a normal animal by the injection of the blood serum of the former into the tissues of the latter; the latter being as a rule at once protected. The antitoxic serums have been employed most frequently to bring about passive immunity. The protective value of diphtheria antitoxin in those that have been exposed to infection is well established. The use of tetanus antitoxin for prophylactic purposes is also recommended in cases where there is a possibility of the development of tetanus.

Vaccination Against Bacterial Diseases.—The employment of various prophylactic vaccinations against infectious diseases has received much attention in recent years. The measures employed in different diseases vary somewhat, though in general the principles are similar.

The first measures of this nature that were employed on a large scale are those of Haffkine in vaccination against

cholera and plague by means of cultures that had been killed by heating to a moderate temperature. Such dead organisms when injected bring about a reaction in the body which is manifested by a marked increase in the specific agglutinative and bactericidal properties of the blood-serum.

Wright introduced a similar method of vaccination against typhoid fever. The prophylactic treatment consists of one or more injections of dead cultures of *bacillus typhosus*.¹

Metchinkoff and Besredka maintain that immunity is less complete and is accompanied by more severe reactions when induced by dead bacterial vaccines than when a small quantity of "sensitized" living culture is employed.

With this in mind they prepare vaccines by subjecting living cultures to the action of specific immune serum that has been heated sufficiently to destroy its disintegrating power. By this plan the haptophore side chains of the bacteria are saturated with specific immune bodies, manifested by the agglutination of the bacteria. The agglutinated mass is then washed to remove the serum, centrifuged and the sediment used for vaccination. The subcutaneous injection of vaccines so prepared is said to be followed by little or no local pain and almost no constitutional reactions. These advantages are attributed to the sensitization *in vitro*, which would otherwise go on within the tissues and account for the undesirable reactions.

The method of Gay differs from the foregoing in that the sensitized living bacteria are killed by heating before they are injected.

¹ See Chapter on Typhoid Fever.

Precipitins.—The immunization of animals with a variety of substances other than bacteria has served further to demonstrate the complex mechanism of immunity. One of the reactions that is noticed as the result of such immunization is the precipitation observed when the serum of the immunized animal is mixed with the substance with which it has been treated. For instance, the serum of an animal that has received repeated injections of blood, tissue juices or certain secretions from alien species, will cause a precipitate to form when mixed with either of these substances *in vitro*. These “precipitins,” as the newly formed bodies in the blood of the treated animal are called, are specific in that they form precipitates only with the materials injected.

This precipitin reaction is so characteristic that it is employed for the identification of blood in medicolegal cases requiring the differentiation between human blood and that of domestic animals; thus, the serum of a rabbit into which human blood has been injected will cause a precipitate with no other blood except that of the anthropoid ape.

In like manner, the repeated injection of milk of one species of animal into the tissues of another will result in the formation of specific precipitins in the blood serum of the treated animal, that will precipitate only the milk of that species of animal from which the milk was derived.

Agglutinins.—If the blood serum of an individual who has recovered from a bacterial infection or who has been rendered immune by bacterial vaccination be mixed with the bacteria that caused the infection or those used in the vaccination—the bacteria, if motile, lose their motility and finally clump together in masses, *i. e.*, they are “agglu-

tinated" by the serum; the reaction being referable to the presence of a new body—"agglutinin"—that has appeared in the blood as a result of the infection or the vaccination. The relation of this newly formed antibody is specific, *i. e.*, it agglutinates only those agents that called it forth. In the normal blood agglutinating activity may often be demonstrated for a variety of bacteria (Bergey) but it is never as high in potency as is that which may be artificially induced, or that seen early in convalescence from a number of infections.

The agglutinating properties of an immune serum are not indicative of the degree of immunity possessed by the individual from whom the blood was drawn. There may be a relatively high degree of agglutinating property with no demonstrable correspondence in germicidal or protective activity. Though no parallelism necessarily exists between the degree of agglutinating and that of germicidal or bacteriolytic activities of an immune serum, it is nevertheless true that both qualities develop as a result of an effort on the part of the tissues to resist infection, and both may represent a response to the same stimulus.

The specificity of the agglutinating reaction has proved of use in the identification of infective bacteria, and conversely, in the recognition of diseases resulting from bacterial invasion. For instance: given an unidentified bacterium of the colon—typhoid—dysentery group that is agglutinated by the serum from a case either of experimentally induced or naturally acquired typhoid fever and is not agglutinated by serum from a dysentery case or one of colon infection—in all human probability that organism is the typhoid bacillus; or given the serum from a patient suffering from an undetermined febrile disease that agglu-

tinates *Bacillus typhosus* and no other organism, that patient in all probability is suffering from typhoid fever. This latter application of the reaction constitutes what is generally known as the Widal reaction.

Immunity: Historic Sketch.—In the course of our studies aimed to secure light on the mechanism of infection, two phenomena are constantly in evidence, notably—first, that not all individuals are susceptible to infection by all pathogenic bacteria, and next, that an individual who has recovered from infection has undergone a change during the course of the disease that, as a rule, renders him insusceptible to subsequent infection by the same species of bacteria. Individuals in either the one or the other state are said to be immune; in the former to be immune by nature, in the latter to have acquired immunity.

In its present development there is no more fascinating subject, and none of broader biological significance than that involving this riddle of immunity. For a quarter of a century it has attracted the attention of the most brilliant investigators in medicine and its cognate fields, and, though much has been learned, it is as yet far from fully elucidated. It is obviously inadvisable in a work of this character to follow in detail the manifold lines of investigation aimed to clear up this matter. We shall content ourselves, therefore, with a statement of the significant results and such discussion of them as may be necessary to indicate their bearings upon the problem.

Knowing as we now do that infection is at bottom a matter of intoxication, and believing, as we are led to do by Ehrlich and his pupils, that intoxication is to be interpreted as a destructive union, in the chemical sense, between the poisons on the one hand and cells or parts

of cells for which they have an affinity on the other, natural resistance or immunity from one or another type of infective organism may be interpreted in several ways, namely—that the naturally immune animal is by nature devoid of those cells or parts of cells for which the poison of the infective organism, from which it is immune, has a specific destructive affinity; or, that the animal is by nature endowed with cells, parts of cells or products of cell life that serve as antidotes for the poison of the infective organism in question; or, again, that certain cells of the immune animal have the power to actually destroy the infective organism when it gains access to the body, thereby not only preventing its growth and multiplication, but simultaneously rendering inert the poisons liberated as a result of its disintegration.

Long before the present state of our knowledge on this subject had been reached, observers who were occupied with the study of infection had offered certain explanations for the occasional failure of their efforts to cause disease by inoculation. In the majority of cases such doctrines or hypotheses were offered in connection with the immunity that had been acquired. This is not surprising, since artificially induced immunity—*i. e.*, acquired immunity—is a constitutional state that is more or less under the control of the experimenter, while natural immunity is an hereditary, idioplasmic peculiarity that can be modified little if at all by any of the known experimental procedures.

Among the first to offer an explanation for the condition of acquired immunity was Chauveau, who, in 1880, suggested that the immunity commonly observed in animals that had recovered from a specific infection, and likewise immunity produced artificially by vaccination, is referable

to a product of the infective organisms that is retained in the tissues, and which, by its presence serves to prevent the development of the same species of organisms should they subsequently gain access to the tissues. This doctrine is usually known as Chauveau's "Retention Hypothesis of Immunity." We shall see later that it is only in small part, if at all, a tenable theory.

As opposed to Chauveau, Pasteur and his pupils, in the same year (1880), expressed the opinion that acquired immunity was to be explained in just the reverse way to that conceived by Chauveau. They believed that in the primary attack of infection something was *extracted* from the tissues by the infecting organisms that was necessary to support the growth of the same species should it subsequently invade the body. This doctrine is known as Pasteur's "Exhaustion Hypothesis" of Immunity, and has apparently little claim to serious consideration.

Four years later (1884) Metchnikoff, while engaged upon the study of certain lower forms of animal life, noticed that particular mesodermal cells, in the course of their wanderings through the body, had the power to actually pick up, insoluble particles that had gained access to it in one way or another. He looked upon them as functioning, therefore, as scavengers. These phagocytes, as they are now generally known, are common not alone to the lower forms of life, but to the most highly organized as well. In the higher forms of animal life, the function of phagocytosis is conspicuously exhibited by the wandering cells—*i. e.*, the white blood corpuscles. In a lower degree the inclusion of foreign bodies with their subsequent digestion or disintegration may occasionally be seen in other cells as well.

Metchnikoff believed this phagocytic power to be the

most important defensive mechanism possessed by the body, and believed both natural and acquired immunity to be referable to it; in the former case regarding it as a natural endowment, in the latter as a function that had been excited by the specific stimulus offered by the organisms or their poisons that were concerned in the primary attack of disease from which the animal recovered, or by the organisms used in purposely exciting a modified form of the disease by one or another of the modes of protective vaccination.

As the phenomenon of phagocytosis could easily be observed under the microscope, and its observation therefore accessible to all interested in the question, the plausibility of the doctrine at once attracted many adherents, and Metchnikoff's views were everywhere accepted as the probable explanation of the defensive mechanism of the body against infection.

In a little while, however, Fluegge, of Breslau, perceiving the incompetency of both Chauveau's and Pasteur's doctrines, observing occasional inconsistencies in Metchnikoff's teaching, and recalling certain significant reactions of the blood that had appeared in the course of experiments by Traube and Gscheidlen, by Fodor, by Rauschenbach, and by Grohmann, determined to subject the whole question to an experimental critical review.

To Nuttall, an American working in his laboratory, was assigned the question of determining if the cell-free blood, or the plasma, was, as had been suggested by Grohmann, possessed of germ-destroying properties. Nuttall's work resulted in a blow to Metchnikoff's doctrine that for a long time seemed to be fatal. He demonstrated that certain virulent bacteria were rendered incapable of development, incapable of infecting susceptible animals, and, in short,

killed by exposure to the serum of animal blood free of all cellular elements. These results naturally caused defections from the ranks of Metchnikoff's followers, especially since Nuttall's deductions were fully confirmed by many distinguished experimentors. In consequence, for a number of years after Nuttall's work, the cell-free fluids of the body were regarded as the real defenses of the body in so far as invading bacteria were concerned.

The natural sequel of Nuttall's demonstration was a general curiosity as to the manner in which the destruction of bacteria was accomplished by the cell-free serum; the conditions that modify the phenomenon; and the nature of the ingredient of the serum to which the germicidal activity might properly be referred.

Buchner demonstrated that active serum was robbed of its germicidal power by dilution with water and by dialysis; that it was not affected by dilution with physiological salt solution; that it was rendered inert by an exposure of fifty minutes to 55° C., and that it was not affected by alternate freezing and thawing. He concluded that the element of the blood to which the function of killing bacteria may be ascribed is a living albumen and suggested "alexin" as the appropriate designation. Hankin and Martin believed the active germicidal principle to be a globulin, a view that was to some extent suggested by the investigations of Ogata and of Tizzoni and Cattani; while the investigations of Vaughan and of Kossel led them to regard nucleins as the most important constituents of the blood in so far as germicidal action is concerned. Fodor believed, as a result of his experiments, that the antibacterial action of the blood could be appreciably accentuated by the addition of alkalies. While Baumgarten and certain of his pupils referred the

death of the bacteria to purely physical conditions; believing that their exposure to blood-serum having an osmotic tension different from the fluids in which they had been growing resulted in disturbances of the bacterial protoplasm that were inconsistent with bacterial life.

By the observations of Behring and Kitasato and of Roux and Yersin entirely new light was thrown upon the subjects of infection and immunity and a new field of inquiry was opened. Through the work of these investigators and their pupils upon tetanus and diphtheria it was demonstrated that immunity was, at least in certain diseases, not so much a matter of actually destroying the invading bacteria as of neutralizing their poisons.

The outcome of these investigations established the fact that if the poisons of tetanus bacilli or of diphtheria bacilli, entirely free from the germs themselves, be injected into susceptible animals in minute sublethal doses the animals presently acquired immunity from both poisons and living organisms. Furthermore, that the blood-serum of animals thus immunized had the power when transferred directly to normal animals of at once rendering them insusceptible to infection by the living germs, and of equal importance, that if the blood-serum of an animal thus immunized be added to the bacteria-free poison of either the tetanus or diphtheria bacillus in a test-tube that the poison was neutralized, *i. e.*, the serum of the animal acted as an antedote which rendered the bacteria poison inert.

It is obvious therefore that through the injections into the normal animals of non-fatal quantities of the specific bacterial poisons the tissues had been stimulated to react in a manner quite in harmony with the views of Buchner expressed in 1883, to the effect that the immunity seen in

an animal that has recovered from a specific infection is explainable by a "reactive change" that has occurred in the tissue cells, as a result of the primary infection or intoxication, which serves to protect the animal from subsequent attacks of a similar character.

The demonstration that the serum of an artificially immunized animal can not only confer immunity upon another animal but, in the case of tetanus and diphtheria in particular, actually cure it after the disease is in progress, is one of the most important steps that has been made in this entire field of inquiry. The triumph resulting from the practical application of this principle to the prevention and cure of diphtheria in man fairly marks an epoch in modern medicine. Though the results attendant upon the application of that principle to the prevention and cure of a number of other diseases—Asiatic cholera, typhoid fever, lobar pneumonia, infection by the pyogenic cocci, rabies, tuberculosis, plague, syphilis, and snake bites—have met with comparatively indifferent success, still the knowledge gained through these efforts has been of inestimable value in stimulating researches that have served to indicate not only the manifold nature of this complex problem but have led to discussions through which some of its most obscure phases have been illuminated.

Briefly stated, the outcome favors the conclusions that the mechanism of immunity varies in different diseases, *i. e.*, that it depends upon the specific peculiarities of the invading bacteria. In some instances it is manifested as an effort on the part of the tissues to neutralize bacterial poisons, the bacteria themselves remaining unaffected; in others as an actual destruction, disintegration or digestion of the invading bacteria together with the neutralization of such intra-

cellular poisons as may be bound up as integral portions of their constituent protoplasm.

Furthermore, in so far at least as induced immunity is concerned, the bulk of the experimental testimony supports the opinion that the reaction is specific; that is to say, be the systemic reaction evidenced as the elaboration of an antidote to a soluble poison or as increased facility to destroy living bacteria, it is called forth only through the specific stimulus afforded by the injection of the animal with the particular poison or bacterium from which we desire to protect it. Thus, for instance, an animal rendered immune from tetanus toxin, is not immune from diphtheria toxin or from the inroads of diphtheria bacilli; similarly an animal immune from any of the pathogenic species of bacteria is immune from that species only and not necessarily from any others.

An observation of fundamental importance to an understanding of the mechanism of immunity was made by R. Pfeiffer in 1895. While investigating Asiatic cholera he found that animals could be immunized from the specific endotoxin of the organism causing that disease; that the blood-serum of such immune animals when injected into normal animals protected them from what would otherwise be a fatal dose of the cholera spirillum; that the peritoneal fluids of the artificially immunized animal had an almost instantaneous bacteriolytic, *i. e.*, disintegrating, action upon living cholera spirilla that were injected directly into the peritoneal cavity; that the serum from the immune animal had no such effect upon cholera spirilla in a test-tube, but if virulent cholera spirilla were injected into the peritoneum of an animal that is *not* immune, and that such injection be followed immediately by an intraperitoneal injection of

blood-serum from an *immune* animal, almost instantly the peculiar disintegration of the bacteria that was observed in the peritoneum of the immune animal was to be seen. As we shall learn presently this observation is of the utmost importance and its bearing upon the course of certain subsequent events will soon be manifest.

The significant features of Pfeiffer's observation are that while the blood serum of an immune animal is capable of conferring immunity upon a susceptible animal, yet, in a test-tube it exhibits none of the bacteriolytic activity constantly to be noted in the body of the immune animal; on the other hand if a small quantity of it be injected into the peritoneal cavity of a normal, susceptible animal, the phenomenon of bacteriolysis, hitherto absent, at once makes itself manifest. Clearly the serum requires the coöperation of something within the body of the living animal to bring about the disintegration of bacteria. The phenomenon must therefore be the result of a composite function.

Though Nuttall's work materially lessened the number of adherents to the phagocytic doctrine of Metchnikoff there was still a group of active workers who retained their belief in the fundamental soundness of the idea. Metchnikoff himself never swerved. Without entering into a discussion of the many instructive investigations upon the questions of phagocytosis it will suffice for our purposes to state briefly their culmination. We now know, through the studies notably of Bail and of Kikuchi that on the one hand phagocytosis may be inhibited, and by the demonstrations of Wright and Douglass, in particular, that, on the other, it may be accentuated. Bail, believing the real defenses of the body to be cellular, attributes the failure of the cells to protect from infection to an inhibition of

their defensive powers by a substance, "aggressin," elaborated by the invading bacteria. While Kikuchi, accepting the "aggressin" doctrine, restricts the action of "aggressin" to the leukocytes and interprets it as in the nature of a negative chemotactic phenomenon, whereby the leukocytes are so repelled that they cannot approach and take up the bacteria.

The efforts of Wright and Douglass have been in the way of accentuating phagocytic activity and their results have shed a flood of most important light upon the subject. In 1903 and 1904, in papers presented to the Royal Society of London, they express the opinion that leukocytes alone are incapable of taking up bacteria, and that in order for them to exhibit this function the bacteria must first be acted upon by a something contained in the normal blood, a state of affairs analogous to that observed by Pfeiffer. They conceived this preparation of bacteria for ingestion by leukocytes to be in the nature of the preparation of food for consumption. They employ the term "Opsonin," (meaning to cater for; to prepare food) in designation of the element in the blood having that property. Prior to the observations of Wright and his associates it had been known that if white blood-cells be washed free of all adhering serum they are incapable of taking up bacteria, but the interpretation, in the light of Wright's work, seems to be incorrect. It was believed that a something in the blood, a "stimulin" as it was called by some, acted not on the bacteria but on the leukocytes, stimulating them to activity. Wright and his colleagues have clearly shown the error of that view and have convincingly demonstrated that it is the action of their "opsonin" on the bacteria that makes phagocytosis possible. Thus, for instance, if bacteria and

washed leukocytes be brought together the bacteria are not taken up by the cells; if on the other hand a drop of normal serum be added, phagocytosis begins. Or, if bacteria be immersed in normal serum and then carefully cleaned of all adherent serum by washing they will readily be taken up by leukocytes, even those also freed of all serum by careful washing. In short the action of the serum on the bacteria, through its "opsonin," has been to make them ingestible or digestible for the leukocytes.

This opsonizing property of the blood varies. Under conditions depressing general health it may be diminished; while in the course of infective diseases it is sometimes lessened, sometimes increased. It may be increased by immunization.

The nature of opsonin (or opsonins) is not known. It has been suggested that they are allied to the enzymes. They are destroyed by heat. They may be absorbed entirely from the blood by bacteria with which they combine. They are unstable, becoming gradually inert after withdrawal from the body.

In consequence of these later investigations the phagocytes are again to the fore as one at least of the important defenses of the body and certainly, in so far as the destruction of invading bacteria is concerned, many have come to look upon them as, after all, just what Metchnikoff originally regarded them, the true scavengers of the body.

Though the destruction of bacteria by the fluids of the body had been demonstrated; though their inclusion and digestion by phagocytes could readily be observed; though an antidote for certain of their poisons could be demonstrated in the blood of immunized animals, there was still

wanting an explanation of the mechanism through which these interesting phenomena were accomplished.

Omitting a group of highly suggestive observations made by many competent investigators, we encounter the most elaborate and at the same time the most fascinating effort to interpret the nature of the reactions occurring in the induction of immunity as well as those fundamentally accountable for the natural condition.

To the genius of Ehrlich¹ we owe the "side chain" or "lateral band" theory (Seitenkettentheorie) of immunity.

Its fundamental features comprise the acceptance of Weigert's doctrine concerning the mechanism of physiological tissue-equilibrium and repair; and the assumption of a specific combining relation, or affinity, between toxic substances and the cells of particular tissues.

At the meeting of German Naturalists and Physicians held at Frankfort-on-the-Main, in 1896, Weigert² advanced an hypothesis the essential features of which are that physiological structure and function depend upon the equilibrium of the tissues maintained by virtue of mutual restraint between its component cells; that destruction of a single integer or group of integers of a tissue or a cell removes a corresponding amount of restraint at the point injured, and, therefore, destroys equilibrium and permits of the abnormal exhibition of bioplastic energies on the part of the remaining uninjured components, which activity may be viewed as a compensating hyperplasia; that hyperplasia is not therefore the *direct* result of external irritation, and cannot be, since the action of the irritant is destructive and

¹ Klinisches Jahrbuch, 1897, Bd. vi, Heft 2, S. 300.

² Neue Fragestellungen in der pathologischen Anatomie, Verhandlungen der Ges. deutscher Naturforscher und Aerzte, 1896, S. 121.

is confined to the cells or integers of cells that it destroys, but occurs rather indirectly as a function of the surrounding uninjured tissues that have been excited to bioplastic activity through the removal of the restraint hitherto exerted by the cells destroyed by the irritant; and, finally, when such bioplastic activity is called into play there is always *hypercompensation*—*i. e.*, there is more plastic material generated than is necessary to compensate for the loss. Ehrlich applies this idea to the individual cell, which he conceives to be a complex molecule, comprising a primary central nucleus to which are attached by side chains its secondary atom-groups, in much the same way that our conception of the reaction-structure of complex organic chemical compounds is represented graphically. Injury to one or more of these physiologically essential atom-groups results, according to the view of Weigert, in disturbance of the cell-equilibrium and consequent effort on the part of the surrounding atom-groups at compensatory repair. With this liberation of bioplastic energy there is more plastic material generated than is necessary for the repair of the injury. The excess of this material finds its way into the blood and, as we shall see presently, is regarded by Ehrlich as the real antidotal, immune, or protective substance.

Assuming a specific combining relation between toxic substances and particular cells or secondary atom-groups of cells—and there are experimental grounds for this assumption¹—it is evident that the combination between the intoxicant and the particular atom-group for which it has a specific

¹ See Wassermann und Takaki, Ueber tetanus antitoxische Eigenschaften des normalen Centralnervensystems, Berliner klin. Wochenschrift, 1898, No. 1, S. 5. Neisser und Wechsberg, Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xxxvi, S. 299. Madsen, *ibid.*, Bd. xxxii, S. 214.

affinity is indirectly the cause of compensatory bioplastic activity on the part of similar surrounding atom-groups that have not been destroyed. This results, as we learned above, in hypercompensation, the excess of plastic material being disengaged from the parent-cell and thrown free into the circulating fluids, there to combine directly with the same intoxicant should it subsequently gain access to the animal. This excess of plastic material thrown into the circulation combines, according to Ehrlich,¹ directly with the intoxicant to form physiologically inactive "toxin—antitoxin" compounds, and can therefore be reasonably regarded as the *antitoxic* material of animals rendered immune from bacterial and other toxins.

Since the announcement of that doctrine many important advances have been made in our knowledge of the subject. We have learned that the reactions of immunity or tolerance may be induced by the use of other intoxicants than those elaborated by bacteria, and by the employment of other cells and cell secretions. It has been demonstrated that antibodies, differing in their specific actions from antitoxins, but originating probably in a similar manner, are to be detected in the fluids of animals thus immunized or rendered tolerant. For a long time we have known of the germicidal action of normal blood-serum; since 1895 we have been familiar with the singular bacteriolytic phenomenon demonstrated by Pfeiffer in the peritoneum of animals immune from cholera; later we learned that the development of immunity from a variety of infections is accompanied by a power on the part of the serum of the immune animal to agglutinate the bacteria causing the infection; the work

¹ Zur Kenntniss der Antitoxinwirkung, Fortschritte der Medicin, 1897, Bd. xv, No. 2.

of Wright upon his opsonic doctrine has finally placed the leukocyte among the important defenses of the body and the profoundly interesting investigations of Bordet, Moxter, von Dungern, Fish, and others, have shown that immunity reactions may be induced with cells and secretions of animal origin hitherto regarded as non-irritating and harmless. For instance, we have long known that the blood of one animal may cause fatal intoxication when injected into an animal of different species; but later we learned if that blood be repeatedly injected in non-fatal amounts, the animal receiving the injections after a while becomes tolerant, and its serum reveals the property not only of robbing the alien blood of its hurtful properties, but also of actually dissolving its corpuscles in a test-tube (hemolysis). In an analogous way, if such tissue-cells as epithelium or spermatozoa be injected repeatedly into the tissues of animals, the serum of the blood of those animals acquires the power of agglutinating and finally dissolving (digesting) such cells outside the body; and if so inert a secretion as milk be injected into the tissues, the blood-serum of the animal receiving the injections after a time reacts specifically with that milk in a test-tube—*i. e.*, precipitates it.

From the foregoing we see that in the numerous phases and expressions of this physiological possibility there are produced antibodies having functions totally different from those attributed by Ehrlich to antitoxins—*i. e.*, we have “lysins,” “agglutinins,” “precipitins,” “aggressins,” “opsonins,” etc., that in their mode of action suggest ferments with specific affinities. It is evident that when broadly conceived the mechanism of immunity comprehends very much more than the neutralization of a bacterial toxin by an antitoxin; and, what is more to the point, in many

of these conditions of immunity or tolerance above noted antitoxins, as we know them, are not present at all.

In an important series of papers on the hemolysins published by Ehrlich and Morgenroth¹ an effort is made to elucidate further the finer mechanism of immunity in its broad sense and various expressions, and to adapt the side-chain doctrine to those more complicated phenomena in which immunity depends not only on the elaboration of antitoxins, but also upon a power on the part of the animal fluids to cause a complete metamorphosis or disappearance of such particulate matters as bacterial and other irritating or poisonous cells and substances. They believe the forces at work in the establishment of immunity from bacteria and from bacterial and other toxins, those operative in the elaboration of the newly discovered lysins, antilysins, agglutinins, precipitins, ferments, antiferments, etc., as well as those concerned in physiological assimilation and nutrition, to be fundamentally identical. They believe susceptibility to infection, as well as power to assimilate nutrition, to be explainable through the assumption that special molecular groups of the living protoplasm are endowed with specific combining affinities for particular matters; and in so far as the establishment of disease is concerned, they regard the receptivity of the individual to be determined entirely by the greater or less susceptibility of those protoplasmic molecular groups—"receptors," as they designate them—to disease-producing agents. In individuals that have been artificially immunized from hurtful substances they believe (in reiteration of Ehrlich's view expressed

¹ Berliner klinische Wochenschrift, 1899, Bd. xxxvi, S. 6 and 481; 1900, Bd. xxxvii, S. 458 and 681; 1901, Bd. xxxviii, S. 251, 569, 598. See also Schlussbetrachtung: Ehrlich in Nothnagel's Speciellen Pathologic und Therapie, Bd. vii, Theil 1, Heft 3, S. 161.

above) that the receptive molecules have been more or less multiplied, according to the degree of immunity, through bioplastic activity of similar, unimpaired atom-groups surrounding those more directly influenced by the intoxicant during the process of immunization; and that this excess of such "receptors," although physiologically useless, being of no known service to normal function, circulates unchanged in the blood, and serves, through specific combining affinity for the poison against which the animal has been rendered immune, to protect the normal tissues from its hurtful action.

According to the nature of the irritant from which the animal has been immunized, the "receptor" is conceived to be either of simple or complex construction, and its protective function to be performed in either a comparatively simple and direct way, or in a more or less complicated and roundabout manner.

As a result of his studies of toxins, Ehrlich reached the conclusion that they are composed of at least two functionally distinct atom-groups: the one, a "haptophore" group, characterized by its *combining* tendencies; the other, a "toxophore" group, distinguished for its *intoxicating* powers; and that for the exhibition of its hurtful characteristics a toxin molecule needs to be first anchored, so to speak, to the susceptible tissue by the "haptophore" group, after which its intoxicating characteristics are exhibited by the "toxophore" group. He conceives the "receptors" to be likewise provided with "haptophore" groups that pair with the corresponding "haptophores" of the poison to which the animal is susceptible or from which it has been immunized. Where immunization has been induced against such relatively simple substances as toxins, ferments, and certain

cell secretions, the "receptors" and their functions are comparatively simple—*i. e.*, the single haptophore of the simple receptor pairs with that of the intoxicant and a physiologically inert complex results. He conceives antitoxins to be simple receptors of this type, and believes the neutralization of toxins by them to take place in this manner. On the other hand, if the immunization of an animal is accompanied by an acquired power on the part of its serum to disintegrate bacteria, to dissolve alien erythrocytes, to digest such cellular elements as epithelium and spermatozoa, to precipitate milk, or agglutinate bacterial or blood-cells, as the studies of Pfeiffer, Bordet, von Dungern, Moxter, Fish, Belfonte and Carbon, Metchnikoff, Gruber, Durham, Widal, and others have demonstrated, then the process becomes less simple, and the atomic grouping of the receptive molecule is correspondingly more complex. In some cases the receptor is provided with both a haptophore and a ferment-like (zymophore) group; the function of the former being to combine with and hold in close proximity to the latter the albumin molecule that is to be destroyed or assimilated; in this way bringing and holding the albumin molecule directly under the influence of the zymophore group. In other cases the "receptor" functions symbolically, so to speak, with a complementary something that circulates normally in the blood, the so-called "complement" of Ehrlich and Morgenroth. Under these circumstances the "receptor" is conceived to be provided with two "haptophore" groups, and becomes an "amboceptor," therefore, the one haptophore of which takes up and fixes the invading bacteria, tissue-cell, or albumin molecule, while the other pairs with the corresponding haptophore of the complement, fixing the latter in close proximity to the invading body, and

thereby favoring the immediate destructive activity of its "zymotoxic" group.

It is of importance to note in connection with this hypothesis, that both "receptors" and "complement" are present in normal susceptible, as well as in immune animals, but that during immunization only the "receptors" are multiplied as a result of the specific stimulation necessary to the establishment of immunity, hence the commonly employed synonymous designations: "immune bodies" and "antibodies." As such bodies are generated during immunization, the substance used for the purpose is designated "antigen"—*i. e.*, generator of antibodies.

The Origin of Complement.—The origin of complement is a question that is still unsolved. Some investigators are inclined to believe that it is derived from the leukocytes. This is the opinion of Metchnikoff and his associates, while others believe that it is derived from other cells and organs as well as from the leukocytes. Again other investigators believe that it is not derived from the leukocytes at all, but from the cells of certain organs, for instance, the spleen pancreas, liver, and the bone marrow. It is impossible with the knowledge at hand to state definitely the origin of the complement.

On the Specificity of Complement.—According to Ehrlich and his pupils the term "complement" is to be used generically to indicate a group of closely allied bodies differing from one another in that they possess specific relations to particular antigens. By appropriate methods they claim to have demonstrated the multiplicity of complement. They state that by particular treatment one or more complementary bodies may be removed from normal blood while others remain in the blood intact; even by such mechanical pro-

cedures as filtering through porcelain some complements are held back while others pass through with the serum.

On the other hand, evidence afforded by the investigations, particularly of Buchner, and Bordet and his pupils point in the opposite direction so insistently as to justify some doubt of the accuracy of Ehrlich's views. Probably the most important evidence in favor of the unity of complement, as conceived by these investigators, is afforded by the every day tests for fixation of complement (to be described later). In the light of these tests "complement," it seems, must be nonspecific in its physiological activities, therefore it is a unit.

SUMMARY.—According to the nature of the intoxicant from which the individual is immunized, the one or the other of the structurally and functionally different types of receptors is increased—*i. e.*, in immunity from a simple toxin the simplest type of receptor, the antitoxic, appears in the blood (receptors of the first order, Ehrlich); in immunity that is associated with agglutinating or precipitating powers on the part of the blood-serum receptors having a haptophore and a zymophore group appear (receptors of the second order); while in immunity from such molecular complexes as blood-, tissue-, or bacterial cells there are produced receptors of the third order, which act through their haptophore groups as intermediate links between the body to be destroyed and the normally present ferment-like complement that is to bring about the destruction. For all the foreign cellular irritants from which animals have been immunized, be they alien blood, tissue-cells, milk, or bacteria, there is assumed to be circulating normally in the blood "complement" on the one hand, and specific "receptors" on the other. This idea of plurality for the complement

is apparently the vulnerable point in the argument (see above "On the Specificity of Complement"). At all events, it has been vigorously assailed by Bordet and Buchner, especially, who as said above, consider the complement a unit, and who do not regard it as possessed necessarily of specific affinities beyond those common to what may be termed proteolytic enzymes in general; and Buchner regards it as nothing more than the normally present "alexin," to which he called attention years ago, while with equal warrant Wright might regard it as the "opsonin" on which he has made such instructive studies. Whether these objections be well taken or not, whether the doctrine as a whole can be accepted or not, the experimental data on which it is based justify the opinion that it is the only satisfactory working hypothesis that has been offered in explanation of the mechanism of what Buchner years ago designated the "reactive tissue-changes" underlying the establishment of acquired immunity.¹ Ehrlich's conception may be graphically represented as follows:

The observations serving as the basis for this doctrine have given to the blood and fluids of the body a new and peculiar interest. According to circumstances, there may be detected in the blood and tissue-juices a number of molecular complexes having totally different functions and affinities, and therefore presumably different from one another:

First, there is normally present in the blood-serum of practically all animals the defensive "alexins" already mentioned.

¹ Justice cannot be done to the beauty and ingenuity of this conception in so brief a summary as is appropriate to a text-book. To be appreciated it must be read as it came from the authors.

Second, there are the antitoxins, found in the blood of animals artificially immunized from special sorts of intoxication, as well as occasionally in the blood and tissues of normal animals, the functions of which are susceptible of demonstration outside the body as well as within the tissues of the living animal.

Third, a body possessed of disintegrating, bacteriolytic powers, a bacteriolysin—*i. e.*, having the property of actually dissolving bacteria, so that the phenomenon may be observed under the microscope. This phenomenon, generally known as “Pfeffer’s Phenomenon,” is especially to be seen within the peritoneum of guinea-pigs that have been rendered immune from Asiatic cholera and from the typhoid and colon infections and intoxications. It is not to be confounded with the ordinary bactericidal function of the alexins that is demonstrable in most normal serums.

Fourth, a body, the so-called “agglutinin” (Gruber), that was considered by Widal to represent a “reaction of infection,” and not of immunity; though at this time its presence is generally supposed to indicate an effort on the part of the body to resist infection. The presence of this body in a serum of an animal is announced by its peculiar influence on the activity and arrangement of the particular species of bacteria from which the individual is immune, or with which it is infected. In the case of typhoid fever in man, for instance, the serum obtained during the early and middle stages of the disease, when mixed with fluid cultures or suspensions of the typhoid bacillus, causes the bacilli to lose their motility and to congregate (agglutinate) in masses and clumps, a condition never seen in normal cultures of this organism, and practically never observed when normal serum is employed instead of the typhoid serum. The

blood of animals artificially immunized from cholera, pyocyaneus, typhoid, dysentery, and colon infections also show the presence of "agglutinin." So far as experience has gone, this agglutinating property is manifested in the great majority of cases only upon the particular organisms from which the animal supplying the serum is protected; that is to say, the relation is specific. In view of the fact that the power of a serum to agglutinate bacteria is regarded by many as a concomitant of infection, the exhibition of this property by the blood of immune animals may at first sight appear paradoxical. We should not lose sight of the fact, however, that agglutinin is presumably distinct from the other substance concerned in immunity, and its presence in immune animals may, therefore, be reasonably explained as a more or less permanent result of the "reactions of infection" that were coincident with the primary stimulations by specific infective or intoxicating matters necessary to the establishment of the condition of immunity; nor should we in this connection lose sight of the fact that its presence is constantly to be demonstrated in typical cases of typhoid fever, for instance, that terminate fatally, and that have exhibited little or no clinical signs of resistance at any time during their course.

Fifth, there may be demonstrated in the blood of animals that have received repeated subcutaneous injections of milk a body—a "precipitin"—that causes a precipitation of milk. This precipitation represents apparently a specific reaction, for it occurs only when the blood-serum is mixed with milk from the species of animal that supplied the milk used for immunization.

Sixth, after the repeated injection of blood or of emulsions of tissue-cells into the body of an animal, there appear in

the blood of that animal certain solvents, or enzyme-like bodies, "hemolysins," "cytolysins," etc., that react specifically upon the blood or tissue-cells injected, agglutinating, disintegrating, and finally completely dissolving them. Here, too, the relations are specific. If a rabbit, for instance, be rendered tolerant to or immune from beef-blood, its serum dissolves only the red corpuscles of bovines; if from dog's blood, then only the corpuscles of the dog are dissolved by the serum of the rabbit. Similarly, if a rabbit be rendered tolerant to injections of emulsions of epithelium cells, then its serum dissolves epithelium and not necessarily other cells. All these reactions may be seen in a test-tube or under the microscope.

Seventh, if a hemolyzing serum, prepared as indicated under the sixth observation, be heated for a short time to 54° – 56° C., it at once loses the hemolytic function, but regains it again if a few drops of serum from a normal animal be added to it. In this phenomenon of hemolysis Ehrlich's "receptors of the third order" are assumed to be concerned; the heating, without injuring the receptors or immune bodies, destroys the "complement," and thereby checks the process; but the subsequent addition of normal serum supplies fresh "complement," and at once restores the combination necessary to the phenomenon of hemolysis.

Eighth, if blood containing a hemolysin or a cytolysin be repeatedly injected into an animal, antibodies—"anti-lysins"—are formed, and the serum of the animal has the power of robbing a hemolytic serum of its hemolyzing function if mixed with it in a test-tube.

Ninth, if normal blood, containing complement, be injected into the same or another species of animal, anti-

complement is formed, which has the property of inhibiting the action of the complement.

Tenth, if emulsions of dead bacteria be injected into animals, the leukocytes of that animal may gain in power to take up and destroy living bacteria of the same species, a result usually attributed to an increase in the opsonizing power of the blood.

Eleventh, there exists in the blood a body to which Wright has given the name "opsonin," which has the function of so acting upon bacteria that they may be taken up by phagocytes. This preparation of the bacteria by opsonin is regarded as a pre-requisite to phagocytosis.

The foregoing sketch affords but an imperfect idea of the vast amount of labor that has been and continues to be expended upon this many-sided, absorbing topic. Of necessity many important contributions have been omitted, but those noted will serve to illustrate the lines along which the solution of the problem has been approached. As a result of such investigations, our knowledge upon infection and immunity may be summarized as follows:

1. That infection may be considered as a contest between bacteria and living tissues, conducted on the part of the former by means of the poisonous products of their growth, and resisted by the latter through the agency of phagocytic cells and the proteid bodies normally present in and generated by their integral cells.

2. That when infection occurs it may be explained either by the excess of vigor of the bacterial products over the antidotal or protective proteids produced by the tissues, or to some cause that has interfered with the normal activity of the phagocytic cells and production of the protective bodies.

3. That in the serum of the normal circulating blood of many animals there exists a substance that is capable, outside of the body, of rendering inert certain pathogenic bacteria, but which is, however, present in such small quantities as to be ineffective, either for the protection of the animal or for the cure of infection when introduced into the body of another animal already infected.

4. That immunity is most frequently seen to follow the introduction into the body of the products of growth of bacteria that in one way or another have been modified. This modification may be artificially produced in the products of virulent organisms, and then introduced into the tissues of the animal; or the virulent bacteria may be so treated that they are no longer of full virulence, and when introduced into the body of the animal will produce poisons of a much less vigorous nature than would otherwise be the case.

5. That immunity following the introduction of bacterial products into the tissues is apparently due to the formation in the tissues of another body or other bodies that act as antidotes to the poisons, and thereby protect the tissues from their hurtful effects.

6. That this protecting proteid which is generated by the cells of the tissues need not of necessity be antagonistic to the life of the invading organisms themselves, but in some cases must be looked upon more as an antidote to their poisonous products.

7. That immunity, as conceived by Ehrlich, may be either "active" or "passive." According to this interpretation, it is "active" when resulting from an ordinary non-fatal attack of infectious disease; or from a mild attack of infection purposely induced through the use of living

vaccines; or from the introduction of cultures of the bacteria that have been killed by heat; or from the gradual introduction of toxins into the tissues until a marked antitoxic state is reached. It is "passive" when occurring as a result of the direct transference of the perfected immunizing substance from an immune to a susceptible animal, as by the injection of blood-serum from the former into the latter. "Passive immunity" is, in most cases, conferred at once, without the delay incidental to the usual modes of establishing "active immunity." As a rule, "active" is a more lasting than "passive" immunity.

8. That phagocytosis is effective in warding off disease in normal individuals only when the defenses of the body are fully active; when the number of invading bacteria is relatively small or when the bacteria are possessed of low aggressive powers. It is probably a secondary process, the bacteria being taken up by the leukocytes only after having been modified through the opsonizing activity of the serum of the blood and of other fluids in the body.

9. That of the hypotheses advanced in explanation of acquired immunity, the one worthy of greatest confidence is that which assumes immunity to be due to reactive changes on the part of the tissues that result in the formation in these tissues of antitoxic and other antibodies, which circulate free in the blood, and in a variety of ways serve to screen the tissues from the harmful effect of extraneous intoxicants and irritants, in some cases acting principally as antidotes to toxins, in others exhibiting more the germicidal (bacteriolytic) than the antitoxic property.

CHAPTER XV.

Hemolysis—The Hemolytic System—Identification of Specific Immune Bodies and Specific Antigens by Their Ability to Fix Complement—The Wassermann Reaction—Schematic Representation of Reactions.

THE HEMOLYTIC REACTION.

THE term hemolysis relates to a phenomenon through which hemoglobin is caused to escape in solution from red blood corpuscles. The process is also known as "laking." It may be brought about in a number of ways—physical, chemical, and vital. It is with the latter that we are here concerned.

As a result of the investigations of Landois we have known for a long time that the blood of one species of animal often exhibits destructive action upon the corpuscles of the blood of an animal of different species. He showed that grave toxic symptoms, sometimes fatal results, follow upon the introduction of the blood of one species into the veins of another. The blood of the dog is a powerful solvent for the blood corpuscles of many other animals, while that of the horse and of the rabbit has very little solvent action. The corpuscles of the rabbit are readily laked by the blood serum of a number of other species while those of the cat and the dog are much more resistant. The corpuscles of the sheep and of the rabbit are dissolved by dog's serum in a very few minutes.

Landois' investigations explain in a satisfactory way the

fatalities so often attendant upon the earlier practices of transfusion, when it was customary, after a severe hemorrhage, in certain cases of poisoning, especially by carbon monoxide, and in certain pathological states, to transfuse the blood of animals into the veins of man for purposes of resuscitation. So convinced was Landois of the danger attendant upon the practice that he states: the blood of animals should never be transfused into the bloodvessels of man.

For a somewhat shorter time we have known that if such toxic alien blood be injected into animals in non-fatal quantities, that repeated injections of gradually increasing doses may be made until a condition develops in which the receptive animal is immune from the poisonous action of the alien blood. When this point is reached the blood of the immunized animal exhibits specific reactions with the alien blood that are not only of very great theoretical interest, but, as newer developments demonstrate, are susceptible of application to the solution of other problems relating to infection and resistance. Thus, for instance, if a portion of the same blood used in immunizing the animal be repeatedly washed in physiological salt solution until one has nothing left but red-blood corpuscles suspended in salt solution and to this there be added a small amount of the blood serum from the immune animal, and the mixture be allowed to stand for a little while at body temperature, there will be a more or less complete solution of hemoglobin from the washed corpuscles and their stroma will finally collect at the bottom of the vessel as a more or less pale or colorless mass. If instead of using in the experiment the serum just as it comes from the immune animal, we heat it for 30 minutes to 55° C., and then mix it with the same volume of washed corpuscles suspended in salt solution, we find that no solution

of hemoglobin occurs. But, if after a reasonable interval of time we now add to the mixture in which no hemolysis has occurred, a small amount of unheated normal (not immune) serum—hemolysis sets in almost at once and may proceed to completion. Obviously in washing the corpuscles and in heating the serum we have eliminated a factor necessary to hemolysis, which factor is readily supplied by a small quantity of fresh, unheated serum from a non-immune animal.¹

Equally obviously, three factors are concerned in this reaction: blood corpuscles, a something in the serum of the immune animal that is not affected by heating; and a something that is destroyed by the heating.

The heat-proof body is the amboceptor of the third order of Ehrlich or the "immune body"—or the "intermediary body" or the "antibody" as it is severally called. The heat-sensitive body is the "complement" of Ehrlich or the "alexin" of Buchner and Bordet. The blood corpuscles of the alien species represent the "antigen"—*i. e.*, the body which when injected into the animal being immunized stimulates or generates the production of the antibodies, immune bodies or amboceptors as we may choose to call them.

We have already learned that amboceptors, or antibodies of this order are conceived by Ehrlich to possess two haptophore groups; the one having the power to unite with a corresponding haptophore of the "complement," the other with a corresponding side chain haptophore, or combining group, of the body to be destroyed—in this case, the alien blood corpuscles. When this combination is complete the complement by its ferment-like action, destroys the blood

¹ Has this any resemblance to the reaction known as "Pfeiffer's phenomenon?"

corpuscles which have been "sensitized" by their union with the antibodies. Such destruction is not possible until the complement is bound by means of the intermediary body with the other object—the blood corpuscle; neither is such destruction possible when complement is, as we just saw, absent or rendered inert by heat or otherwise. In brief, we have here a "system" the integers of which must all be present and in appropriate adjustment before the desired reaction occurs. The several factors and the reaction may be for convenience of visualization graphically represented, thus:

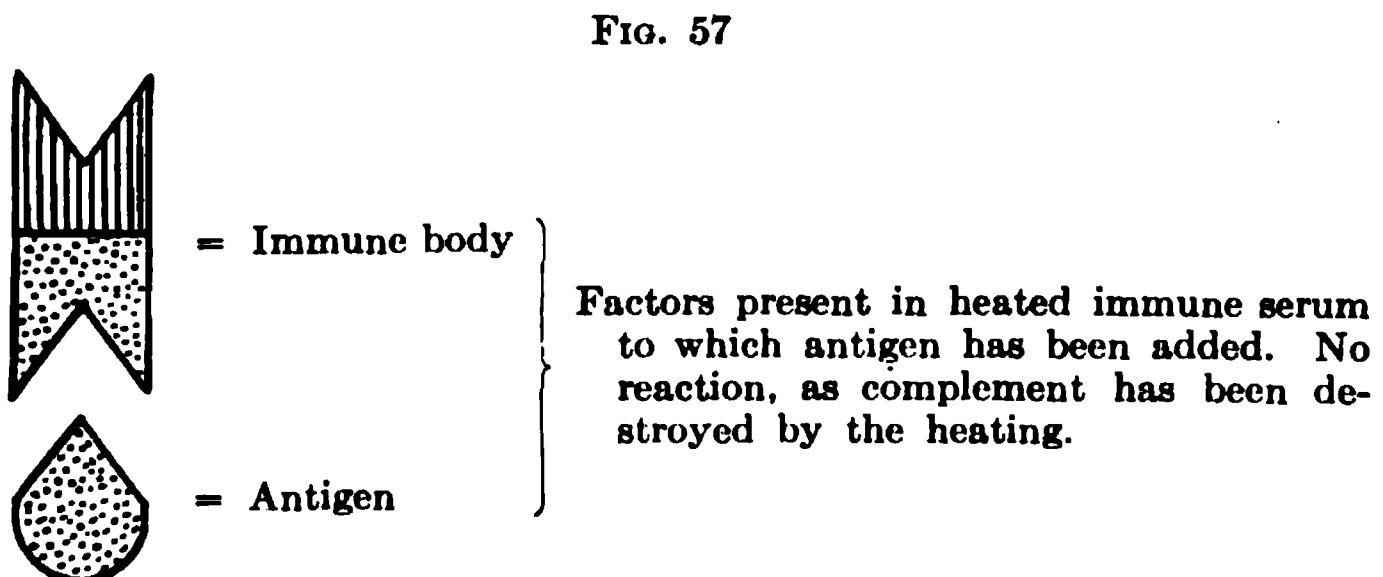
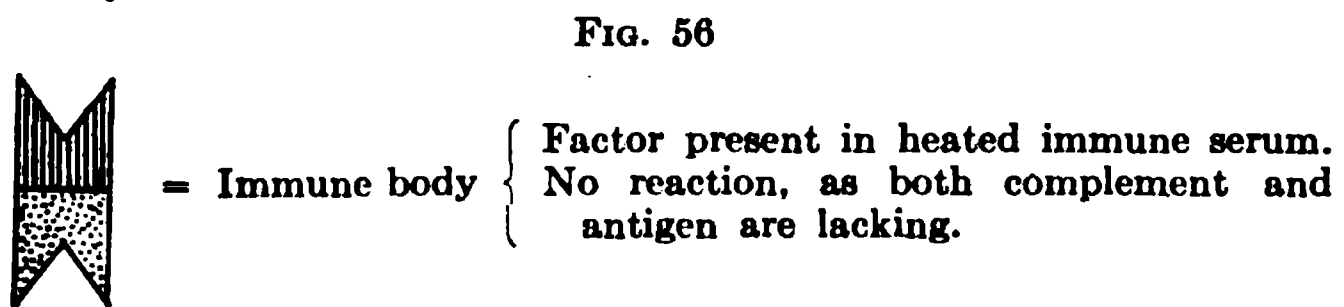
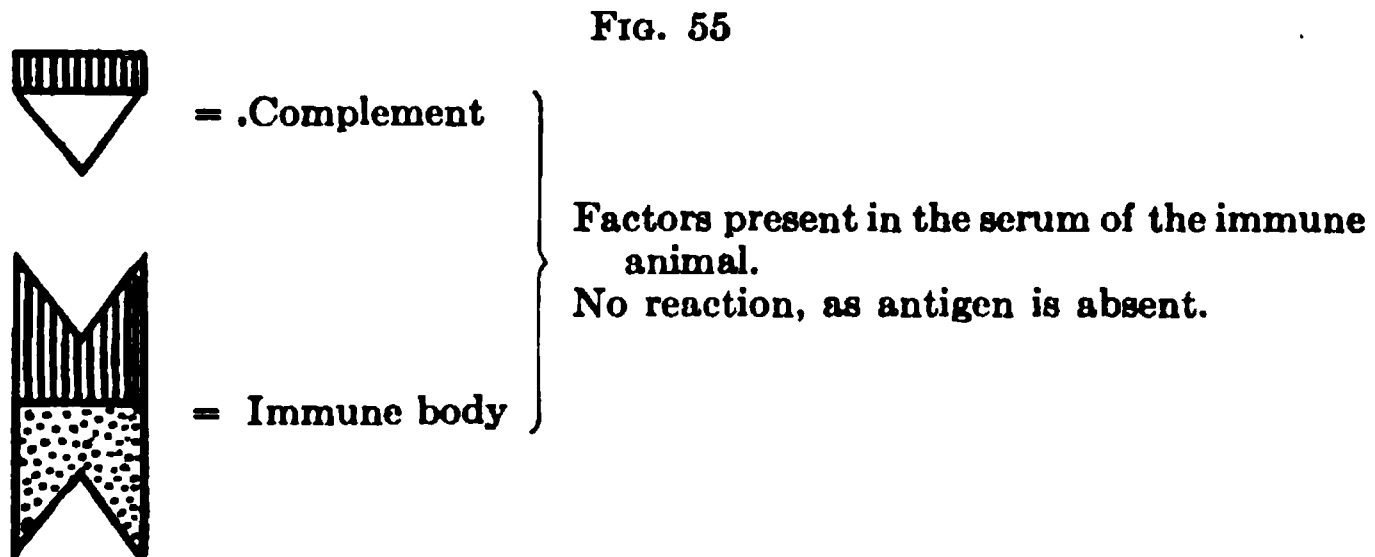
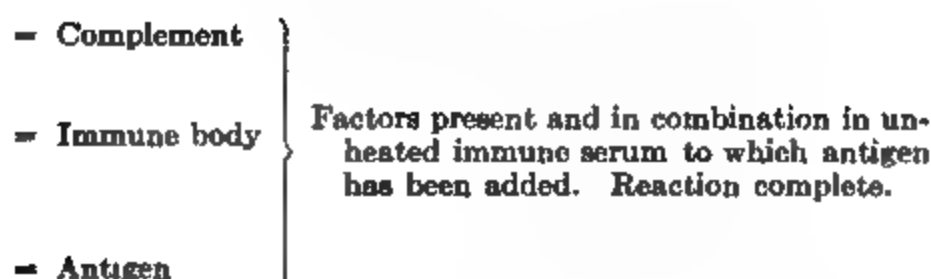


FIG. 58



In the hemolytic system it is obvious, in so far as two factors are concerned, that specific relationship is essential to the reaction. Thus, immune serum from an animal immunized from sheep's blood possesses amboceptors specific for the sheep's blood corpuscles and none for the corpuscles of other animals, so that if to such immune serum blood corpuscles other than those of the sheep be added, no hemolysis occurs, even though it may have been conspicuously active for sheep's corpuscles.

The relationship of the complement to the amboceptor and antigen is not specific. It reacts with any or all amboceptors and antigens and is present in all mammalian blood.

It must not be forgotten, as stated above, that natural hemolytic activity is sometimes exhibited by one blood for another, consequently, in arranging studies in this field this fact should be borne in mind and care exercised to control all experiments.

FIXATION OF COMPLEMENT.

From the investigations of Bordet and Gengou upon the relations between antibodies and complement, methods have been developed by which it is possible to detect very small quantities of antibodies in fluids under question on the one

hand, and to identify, on the other hand, antigens whose true nature may only be suspected.

The important points brought out in their fundamental experiment are: that complement is not specific in its affinities and that when once fixed by an antibody to an antigen the union is not dissociable. The experimental procedures necessary to this demonstration consisted in two series of mixtures—one in which antibody, its antigen and comple-

FIG. 59
SERIES I.

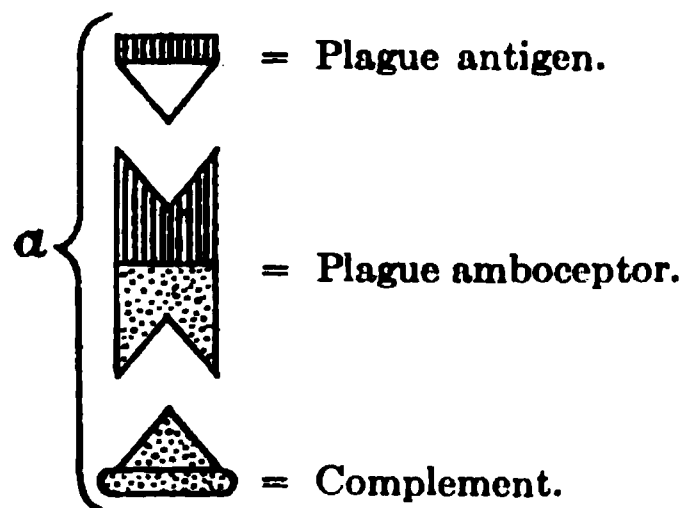
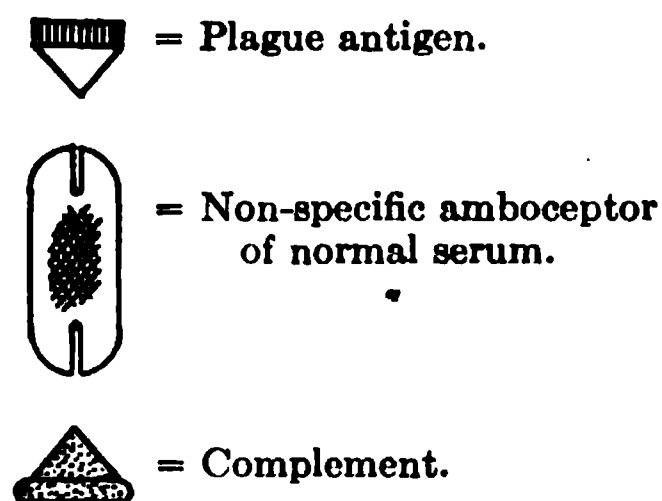


FIG. 60
SERIES II.



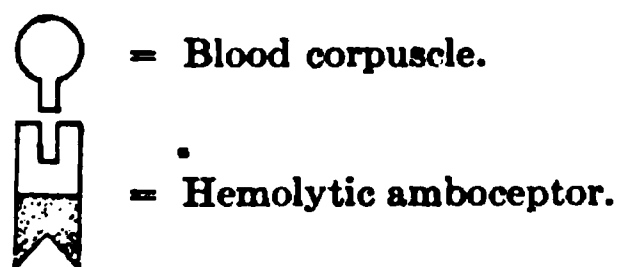
Only two necessary factors present; no union possible.

Washed corpuscles and inactivated hemolytic immune serum now added to each series.

ment were together, the other identical in its ingredients save for the absence of antibodies specific for the antigen used. Figs. 59 and 60. It is obvious that in the first mixture all factors necessary to the saturation of the haptophores of the amboceptor were present—therefore, complement, being one of these factors was bound by the amboceptor to the antigen. In the other mixture this was not possible as there were no amboceptors specific for the antigen in it. But to

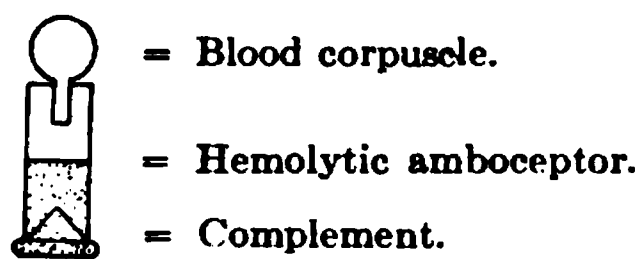
prove this "fixation" of complement in the first mixture: to this end, after the mixtures had stood for a time, an incomplete hemolytic system was added to each mixture—that is, an amount of normal washed blood corpuscles and a portion of inactivated immune serum otherwise hemolytic for those corpuscles, was added. Before this addition, obviously, no hemolysis could occur, because the complement of the hemolytic serum had been destroyed by the heat used for inactivation. But after the addition hemolysis did occur in one tube but not in the other. It is plain that complement necessary to the phenomenon of hemolysis must have been available in one of the tubes. If one recalls that in the second

FIG. 61



No hemolysis. No complement available; all fixed, as in *a'*.

FIG. 62



Hemolysis. Free complement of original mixture now bound by hemolytic amboceptor.

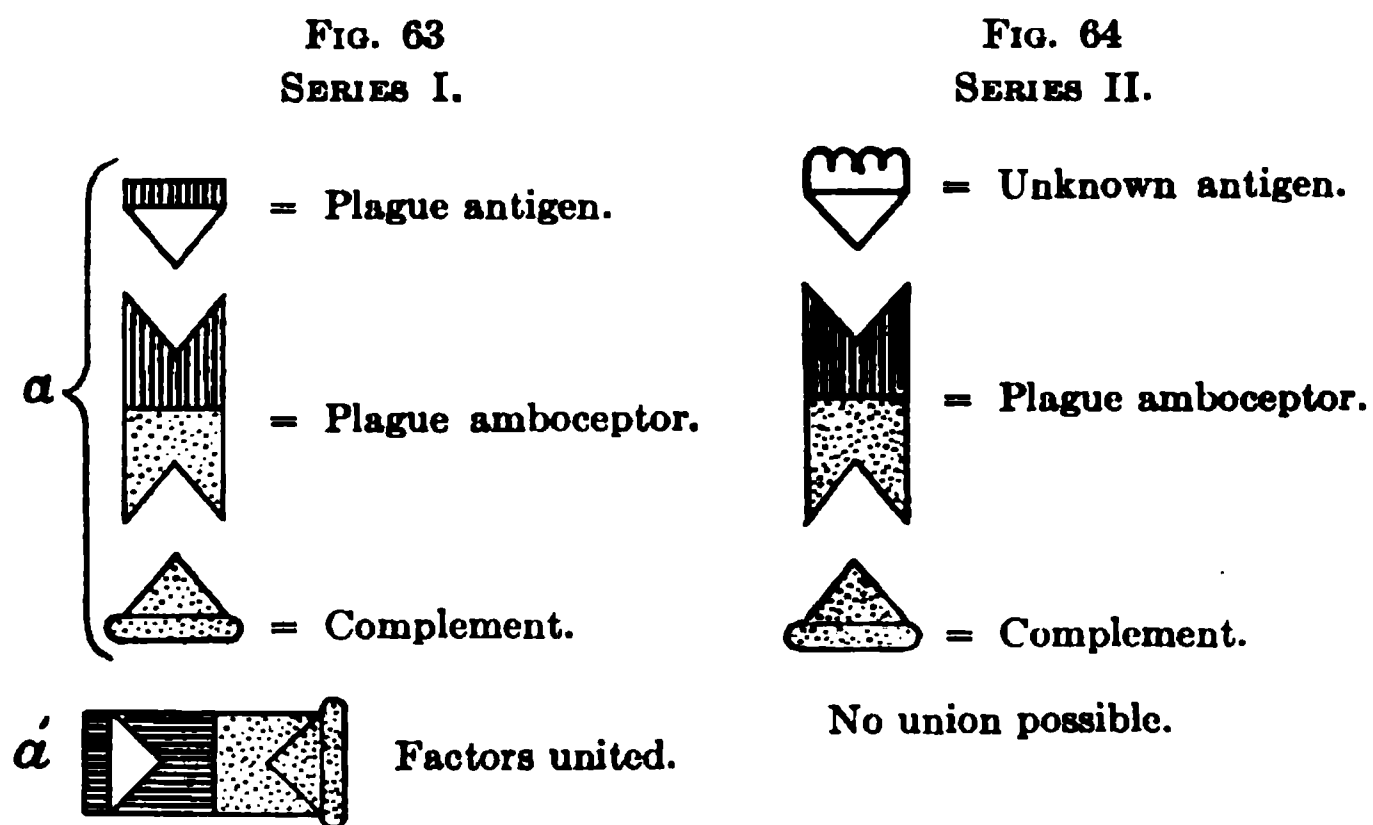
mixture no immune bodies or amboceptors specifically related to the antigen were present it is clear that the complement could not have been bound or fixed. It must have remained free in the serum, available for complementing the action of the hemolytic amboceptors and thereby hemolyzing or destroying the normal blood corpuscles added, as shown by the laking of such corpuscles in the tube. This, in short, is what occurred. See Figs. 61 and 62.

For this particular test, Bordet and Gengou used plague antigen (plague bacilli); plague amboceptors (present in blood of animal immunized from plague); complement (free in normal mammalian blood); normal serum (containing no

specific amboceptors); washed mammalian blood corpuscles and inactivated immune serum hemolytic for such corpuscles (such serum contains only hemolytic amboceptors, no complement).

The application of the principles involved in this experiment to the solution of a number of practical problems is evident. For instance, we are called upon to identify the nature of an obscure infection, latent syphilis, let us say. We know that the blood in such cases contains specific antibodies for the antigen (excitor) of syphilis. We know that the excitor of syphilis or important extractives of it are present in the organs of syphilitic fetuses, so that the antigen is easy to obtain. We know that all normal mammalian blood contains complement. If, therefore, a mixture be made of syphilitic antigen, of normal guinea-pig blood serum and of the patient's blood serum, we have, providing the patient be syphilitic, all the factors necessary to the union of complement to antigen by the amboceptors of the blood. If, after it has stood for a time, we now add to such a mixture hemolytic amboceptors and red corpuscles to which such amboceptors are specifically related, we get no hemolysis, *if the patient be syphilitic*, for there is no free complement left for the completion of the hemolytic system—on the other hand if the patient *be not syphilitic*, his blood will contain no amboceptors capable of binding complement and syphilitic antigen together, therefore, there will be free complement available for the hemolytic system and hemolysis results. The application of this principle to the diagnosis of obscure syphilis constitutes what is generally known as "The Wassermann Reaction," but it is plain that the principle is susceptible of application to the identification of other latent infective processes as well. In fact it is being more and more used for that purpose.

A glance at the graphic representation of this reaction at once also suggests the means of identifying unknown but suspected antigens. Thus, for instance, if in both series we have the same amboceptors and complement but different antigens, one being specifically related to the amboceptor, the other not, plainly we will have a result similar to that obtained in the first series after the incomplete hemolytic system is added—that is, there will be no hemolysis in the tube in which antigen and amboceptor are specifically related, for here all free complement will be fixed—on the other hand in the tube in which the antigen is not so related to the amboceptor complement cannot be so fixed and it, therefore, as in the first experiment, remains free to complete the hemolytic system. The reaction may be expressed graphically as follows:



Washed corpuscles and inactivated hemolytic immune serum now added to each mixture.

In the second application of this test observe that the unknown antigen used in Series II is not of the nature of

plague antigen. Had the problem involved the identification of any other antigen—say gonococci, bacillus mallei or others—one would substitute in the mixtures gonorrhea antibodies or glanders antibodies or others as the case may be, and proceed as above. In these cases, however, such antibodies must be artificially produced in animals that react to gonorrhea or glanders antigens.

In addition to the foregoing the principles involved in these reactions have been employed for the differentiation of closely allied proteins. Such for instance as the differentiation of bloods. For instance, if an animal be immunized from human blood its serum will contain amboceptors for human blood corpuscles, the antigen. Such amboceptors in the presence of human corpuscles or their protein extractives and complement *fix* the complement; on the other hand if the blood under question be from other species than man, no such fixation can occur as there is no specific affinity between such blood and the amboceptors for human blood. Consequently, in the final test for fixation, as determined by + or — hemolysis, no hemolysis occurs after the addition of hemolytic amboceptors and their related corpuscles to the mixture of human blood, its amboceptors and complement, while hemolysis does occur in the mixture of alien blood, human amboceptors, and complement.

In the former case all complement was fixed to the antigen by the homologous amboceptors, while in the latter this was not possible because of the lack of specific affinity of human blood amboceptors for the alien blood.

While the principles involved in the practical application of these reactions are very simple, yet there are so many chances for error that each and every step demands the most careful control.

APPLICATION OF THE METHODS OF BACTERIOLOGY.

CHAPTER XVI.

To Obtain Material with Which to Begin Work.

EXPOSE to the air of an inhabited room a slice of freshly steamed potato or a bit of slightly moistened bread upon a plate for about one hour. Then cover it with an ordinary water-glass, place it in a warm spot (temperature not to exceed that of the human body— 37.5° C.), and allow it to remain undisturbed. In from twenty-four to thirty-six hours there will be seen upon the cut surface of the bread or potato small, round, oval, or irregularly round patches which present various appearances. These differences in macroscopic appearance are due in some cases to the presence or absence of color; in others to a higher or lower degree of moisture; in some instances a patch will be glistening and smooth, while its neighbor may be dull and rough or wrinkled; here will appear an island regularly round in outline, and there an area of irregular, ragged deposit. All these gross appearances are of value in aiding us to distinguish between these colonies—for colonies they are, and under the same conditions the organisms composing each of them will always produce growth of exactly the same appearance. It was just such an observation as this that sug-

gested to Koch a means of separating and isolating in pure cultures the component individuals from mixtures of bacteria, and from it the methods of cultivation on solid media were evolved.

If, without molesting these objects, we continue the observations from day to day, we shall notice changes in the colonies, due to the growth and multiplication of the individuals composing them. In some cases the colonies will always retain their sharply cut, round, or oval outline, and will increase but little in size beyond that reached after forty-eight to seventy-two hours; whereas others will spread rapidly and quickly overrun the surface upon which they are growing, and, indeed, grow over the smaller, less rapidly developing colonies. In a number of instances, if the observation be continued long enough, many of these rapidly growing colonies will, after a time, lose their lustrous and smooth or regular surface and will show here and there elevations, which will continue to appear until the whole surface becomes conspicuously wrinkled. Again, bubbles may be seen scattered through the colonies. These are due to the escape of gas resulting from fermentation, which the organisms bring about in the medium upon which they are growing. Sometimes peculiar odors due to the same cause will be noticed.

Note carefully all these changes and appearances, as they must be employed subsequently in identifying the individual organisms from which each colony on the medium has developed.

If we now examine these colonies upon the bread or potato with a hand-lens of low magnifying power, we will be enabled to detect differences not noticeable to the naked eye. In a few cases we may still see nothing more than a

smooth, non-characteristic surface; while in others minute, sometimes regularly arranged tiny corrugations may be observed. In one colony they may appear as tolerably regular lines, radiating from a central spot; and again they may appear as concentric rings; and if by the methods which have been described we obtain from these colonies their individual components in pure culture, we shall see that this characteristic arrangement in folds, radii, or concentric rings, or the production of color, is characteristic of the growth of the organism under the conditions first observed, and by a repetition of those conditions may be reproduced at will.

So much for the simplest naked-eye experiment that can be made in bacteriology, and which serves to furnish the beginner with material upon which to commence his studies. It is not necessary at this time for him to burden his mind with names for these organisms; it is sufficient for him to recognize that they are of different species, and that they possess characteristics which will enable him to differentiate the one from the other.

Exposure and Contact.—Make a number of plates from bits of silk used for sutures, after treating them as follows:

Place some of the pieces (about 5 centimeters long) in a sterilized test-tube, and sterilize them by streaming steam for one hour or in the autoclave for fifteen minutes at one atmosphere pressure. At the end of the sterilization remove one piece with sterilized forceps and allow it to brush against your clothing, then make a plate from it; draw another piece across a dusty table and then plate it. Suspend three or four pieces upon a sterilized wire hook and let them hang for twenty minutes free in the air, being sure that they touch nothing but the hook; then plate them separately.

Note the results.

In what way do these experiments differ and how can the differences be explained?

Expose to the air six Petri dishes into which either sterilized gelatin or agar-agar has been poured and allowed to solidify; allow them to remain exposed for five, ten, fifteen, twenty, twenty-five, and thirty minutes in a room where no one is at work. Treat a second set in the same way in a room where several persons are moving about. Be careful that nothing touches them, and that they are exposed only to the air. Each dish should be carefully labelled with the time of its exposure.

Do they present different results? What is the reason for this difference?

Which predominate—colonies resulting from the growth of bacteria, or those from common molds?

How do you account for this condition?

Sprinkle a little fine dust over the surface of a plate of sterile gelatin or agar-agar; examine the dust-particles with the microscope immediately after depositing them on the medium, and again after eighteen to twenty-four hours. What differences do you detect? What information of sanitary importance does this give?

Under the description of each of the pathogenic bacteria more or less detailed directions will be found for the discovery and isolation of each of the pathogenic bacteria.

CHAPTER XVII.

Various Experiments in Sterilization by Steam and by Hot Air.

PLACE in one of the openings in the cover of the steam sterilizer an accurate thermometer; when the steam has been streaming for a minute or two the thermometer will register 100° C. Wrap in a bundle of towels or rags or pack tightly in cotton a maximum (self-registering) thermometer; let this thermometer be in the centre of a bundle large enough to quite fill the chamber of the sterilizer. At the end of a few minutes' exposure to the streaming steam remove it; it will be found to indicate a temperature of 100° C.

Closer study of the penetration of steam has taught us, however, that the temperature found at the centre of such a mass may sometimes be that of the air in the meshes of the material, and not that of steam, and for this reason the sterilization at that point may not be complete, because hot air at 100° C. has not the sterilizing value that steam has at the same temperature. It is necessary, therefore, that this air should be expelled from the meshes of the material and its place taken by the steam before sterilization is complete. This is insured by allowing the steam to stream through the substances a few minutes before beginning to calculate the time of exposure. There is as yet no absolutely sure means of saying that the temperature at the centre of the mass is that of hot air or of steam, so that the exact length of time that is required for the expulsion of the air from the meshes of the material cannot be given.

Determine if the maximum thermometer indicates a temperature of 100° C. at the centre of a moist bundle in the same way as when a dry bundle was employed.

To about 50 c.c. of bouillon add about 1 gram of chopped hay, and allow it to stand in a warm place for twenty-four hours. At the end of this time it will be found to contain a great variety of organisms. Continue the observation, and ultimately a pellicle will be seen to form on the surface of the fluid. This pellicle is made up of rods which grow as long threads in parallel strands. In many of these rods glistening spores will be seen. After thoroughly shaking, filter the mass through a fine cloth to remove coarser particles.

Pour into each of several test-tubes about 10 c.c. of the filtrate. Allow one tube to remain undisturbed in a warm place. Place another in the steam sterilizer for five minutes; a third for ten minutes; a fourth for one-half hour; a fifth for one hour.

At the end of each of these exposures inoculate a tube of sterilized bouillon from each tube. Likewise make a set of plates or Esmarch tubes upon both gelatin and agar-agar from each tube, and note the results. At the same time prepare a set of plates or Esmarch tubes on agar-agar and on gelatin from the tube which has not been exposed to the action of the steam.

The plates or tubes from the unmolested tube will present colonies of a variety of organisms; separate and study these.

Those from the tube which has been sterilized for five minutes will present colonies in moderate numbers; but, as a rule, they will represent but a single organism. Study this organism in pure cultures.

The same may be predicted for the tube which has been

heated for ten minutes, though the colonies will be fewer in number.

The thirty-minute tube may or may not give one or two colonies of the same organism.

The tube which has been heated for one hour is usually sterile.

The bouillon tubes from the first and second tubes which were heated will usually show the presence of only one organism—the bacillus which gave rise to the pellicle-formation in the original mixture. This organism is *bacillus subtilis*. It is especially adapted to the study of those various degrees of resistance to heat that spore-forming bacteria exhibit at different stages of their development.

Inoculate about 100 c.c. of sterilized bouillon with a very small quantity of a pure culture of this organism, and allow it to stand in a warm place for about six hours. Now subject this culture to the action of steam for five minutes; it will be seen that sterilization, as a rule, is complete.

Treat in the same way a second flask of bouillon, inoculated in the same way with the same organism, but after having stood in a warm place for from forty-eight to seventy-two hours—that is, until spores have formed—and it will be found that sterilization is not complete: the spores of this organism have resisted the action of steam for five minutes.

To determine if sterilization is complete always resort to the culture methods, as the macroscopic and microscopic methods are deceptive; cloudiness of the media or the presence of bacteria microscopically does not always signify that organisms possess the property of life.

Inoculate in the same way a third flask of bouillon with a *very small* drop from one of the old cultures upon which the pellicle has formed; mix it well and subject it to the

action of steam for *two* minutes; then place it to one side for from twenty to twenty-four hours, and again heat for two minutes; allow it to stand for another twenty-four hours, and repeat the process on the third day. No pellicle will be formed, and yet spores were present in the original mixture, and, as we have seen, the spores of this organism are not killed by an exposure of five minutes to steam. How can this result be accounted for?

Saturate several pieces of cotton thread, each about 2 cm. long, in the original decomposed bouillon, and dry them carefully at the ordinary temperature of the room; then at a little higher temperature—about 40° C.—to complete the process. Regulate the temperature of the hot-air sterilizer for about 100° C., and subject several pieces of this infected and dried thread to this temperature for the same lengths of time that we exposed the same organisms in bouillon to the steam, viz., five, ten, thirty, and sixty minutes. At the end of each of these periods remove a bit of thread, and prepare a set of plates or Esmarch tubes from it. Are the results analogous to those obtained when steam was employed?

Increase the temperature of the dry sterilizer and repeat the process. Determine the temperature and time necessary for the destruction of these organisms by dry heat. These threads should not be simply laid upon the bottom of the sterilizer, but should be suspended from a glass rod, which may be placed inside the oven, extending across its top from side to side.

Place several of the infected threads in the centre of a bundle of rags. Subject this to a temperature necessary to sterilize the threads by the dry method. Treat another similar bundle to sterilization by steam. In what way do the results of the two processes differ?

CHAPTER XVIII.

Methods of Testing Disinfectants and Antiseptics—Experiments Illustrating the Precautions to be Taken—Experiments in Skin-disinfection.

DETERMINATION OF DISINFECTANT PROPERTIES.

THERE are several ways of determining the germicidal value of chemical substances, the most common being to expose organisms dried upon bits of silk thread to the disinfectant for different lengths of time, and then, after removing, and carefully washing the threads in water, to place them in nutrient media at a favorable temperature, and notice if any growth appears. If no growth results, the disinfection is presumably successful. Another method is to mix fluid cultures of bacteria with the disinfectant in varying proportions, and, after different intervals of time, to determine if disinfection is in progress by transferring a portion of the mixture to nutrient media, just as in the other methods of work.

By the first of these processes the bits of thread, usually about 1 to 2 cm. long, are placed in a dry test-tube provided with a cotton plug and carefully sterilized, either by the dry method or in the steam sterilizer, before using. They are then immersed in a pure bouillon culture or in a salt-solution suspension of the organism upon which the disinfectant is to be tested. I say "pure culture," because it is always desirable in testing a substance to determine its germicidal value for several different resistant species of

bacteria, both in the vegetating and in the spore stage, and also because it is only by the use of pure cultures of *familiar* species that it is possible to distinguish between the colonies growing from the individuals that have not been destroyed by the disinfectant under investigation and those of unknown species that may appear upon the plate as contaminations occurring during the manipulation.

After the threads have remained in the culture or suspension for from five to ten minutes they are removed under aseptic precautions and carefully separated and spread upon the bottom of a sterilized Petri dish, which is then placed either in the incubator at a temperature not exceeding 38° C. until the excess of fluid has evaporated, or in a desiccator over sulphuric acid, calcium chloride, or any other drying-agent. The threads are not left there until *absolutely* dry, but only until the *excess* of moisture has evaporated. When sufficiently dry they are immersed in solutions of the disinfectant of different but known strengths for a fixed interval of time, say one or two hours, after which they are removed, rinsed in sterilized distilled water to remove the excess of disinfectant adhering to them, and placed in fresh, sterile culture-media, which are then placed in the incubator at from 37° to 38° C. If after twenty-four to forty-eight or seventy-two hours a growth occurs at or about the bit of thread, and if this growth consists of the organism with which the test was made, manifestly there has been no disinfection; if no growth occurs after, at most, ninety-six hours, it is safe to presume that the bacteria have been killed, unless our efforts at rinsing off the excess of disinfectant from the thread have not been successful, and a small amount of disinfectant is still active in preventing development—*i. e.*, is acting as an antiseptic.

By the process in which cultures or suspensions of the organisms are mixed with different but known strengths of the disinfectant a small portion of the mixture, usually a loopful or a drop, is transferred at the end of a definite time to the fresh medium which is to determine whether the organisms have been killed or not. This is commonly a tube of fluid agar-agar, which is poured into a Petri dish, allowed to solidify, and placed in the incubator, as in the preceding method.

After the *minimum strength* of disinfectant necessary to destroy the vitality of the organisms with which we are working has been determined for any fixed time, it remains for us to decide what is the *shortest time* in which this strength will have the same effect. We then work with a constant dilution of the disinfectant, but with varying intervals of exposure—one, five, ten minutes, etc.—until we have decided not only the minimum amount of disinfectant required for the destruction of the bacteria, but the shortest time necessary for this under known conditions.

A factor not to be lost sight of is the temperature at which these experiments are conducted, for it must always be borne in mind that the action of a disinfectant is usually *more energetic* at a *higher* than at a lower temperature.

Now in both of these methods it is easy to see that unless special precautions are taken a minute portion of the disinfectant may be carried along with the thread, or drop, into the medium which is to determine whether the organisms do or do not possess the power of growth, and there have a restraining or antiseptic action. For organisms in their normal condition—that is, those which have never been exposed to the action of a disinfectant—the amount of certain disinfectants that is necessary to restrain growth

is very small indeed; but for organisms that have already been exposed for a time to such agents this amount is very much less. It is plain, then, that if the test is to be an accurate one, precautions must be taken against admitting this minute trace of disinfectant to the medium with which we are to determine whether the bacteria exposed to the disinfectant were killed or not. The precautions hitherto taken to prevent this accident have been, when the threads were employed, washing them in sterilized distilled water and then in alcohol; or, where fluid cultures were mixed with the disinfectant in solution, an effort was usually made to dilute the amount of disinfectant carried over, to a point at which it lost its inhibiting power.

While such precautions are sufficient in many cases, they do not answer for all. Certain chemicals have the property of combining so firmly with the threads upon which the bacteria are located as to require other special means of ridding the threads of them; and in solutions in which proteid substances are present along with the bacteria a similar union between them and the disinfectant may likewise take place. In both instances this amount of disinfectant adhering to the threads or in combination with the proteids must be eliminated, otherwise the results of the test may be fallacious. A partial solution of the problem is given through studies that have been made upon corrosive sublimate in its various applications for disinfecting purposes, and in this connection it has been shown by Shaefer¹ that it is impossible to rid silk threads of the corrosive sublimate adhering to them by simple washing, as the sublimate acts as a mordant and forms a firm union with the

¹ Berliner klin. Wochenschrift, 1890, No. 3, p. 50.

tissues of the threads. Braatz¹ found the same to hold good for catgut. For example, he found that catgut which had been immersed in solutions of corrosive sublimate gave the characteristic reactions of the salt after having been immersed for five weeks in distilled water which had been repeatedly renewed. Braatz remarks that a similar combination between sublimate and cotton will take place after a long time; but it occurs so slowly that it cannot interfere with disinfection-experiments in the same way that silk does.

The most successful attempt at removing all traces of sublimate from the threads or from the proteid substances in which are located the bacteria whose vitality is to be tested was made by Geppert, who subjected them to the action of ammonium sulphide in solution. By this procedure the mercury is converted into inert, insoluble sulphide, and has no inhibiting effect upon the growth of those bacteria that did not succumb to its action when in the form of the bichloride.

Another plan that has been successfully used is to dry the bacteria on small particles of sterile glass rod or on sterile glass beads instead of on threads. The advantages of the method are obvious, but the handling, especially the washing, must be done carefully or all the bacteria will be removed from the glass surfaces.

In the second method of testing disinfectants mentioned above—that is, when cultures of bacteria and solutions of the disinfectant are mixed, and after a time a drop of the mixture is removed and added to sterile nutrient media—the inhibiting amount of disinfectant can readily be got rid of by dilution; that is to say, instead of transferring the

¹ Centralblatt für Bakteriologie und Parasitenkunde, Bd. vii, No. 1, p. 8.

drop directly to the fresh medium, add it to 10 or 12 c.c. of sterilized salt-solution (0.6–0.7 per cent. of NaCl in distilled water) or distilled water, and after thoroughly shaking add a drop of *this* to the medium in which the power of development of the bacteria is to be determined.

Another important point to be borne in mind in testing disinfectants is the necessity of so adjusting the conditions that each individual organism will be exposed to the action of the agent used. When clumps of bacteria exist we are not always assured of this, for only those on the surface of the clump may be affected, while those in the centre of the mass may escape, being protected by those surrounding them. These clumps and minute masses are especially liable to be present in fluid cultures and in suspensions of bacteria, and must be eliminated before the test is begun, if this is to be made by mixing them with solutions of the agent to be tested. This is best accomplished in the following way: the organisms should be cultivated in bouillon containing sand or finely divided particles of glass; after growing for a sufficient length of time they are to be shaken thoroughly, in order that all clumps may be mechanically broken up by the sand. The culture is then filtered through a tube containing closely packed glass-wool.

The filtration may be accomplished without fear of contamination of the culture by the employment of an Allihin tube, which is practically a thick-walled test-tube drawn out to a finer tube at its blunt end so as to convert it into a sort of cylindrical funnel. The tube when ready for use has the appearance shown in Fig. 65.

This tube, after being plugged at the bottom with glass-wool (*a*, Fig. 65), and at its wide extremity with cotton-wool, is placed vertically, small end down, into an Erlen-

meyer flask of about 100 c.c. capacity and sterilized in a steam sterilizer for the proper time. It is kept in the sterilizer until it is to be used, which should be as soon as possible after sterilization.

FIG. 65

The watery suspension or bouillon culture of the organisms is now to be filtered repeatedly through the glass-wool into sterilized flasks until a degree of transparency is reached which will permit the reading of moderately fine print through a layer of the fluid about 2 cm. thick—i. e., through an ordinary test-tube full of it. This filtrate can then be subjected to the action of the disinfectant. As a rule, the results are more uniform than when no attention is paid to the presence of clumps. It is scarcely necessary to say that in the practical employment of disinfectants outside the laboratory no such precautions are taken; but in laboratory work, where it is desired to determine *exactly* the value of different substances as germicides, all the precautions mentioned will be found essential to precision.

The disinfectant value of gases and vapors is determined by their action upon test-objects in closed chambers. The object is to determine the proportion of the gas, when mixed with air, that is required to destroy the bacteria exposed to its action in a given time. For this purpose the test is usually made

Cylindrical funnel used for filtering cultures on which disinfectants are to be tested.

as follows: under a sterilized bell-glass of known capacity the test-objects are placed. Into the chamber is then admitted sufficient of a mixture of air and the gas under consideration, of known proportions, to displace completely all the air; or the pure gas itself may be introduced in amount necessary to give the desired dilution when mixed with the air in the chamber. At the expiration of the time decided upon for the test the infected articles are removed and the vitality of the bacteria upon them is determined.

In the case of vapors of volatile fluids, such, for instance, as formalin, the fluid is placed under the bell-glass in an open dish; in another open dish the test-objects are placed. The bell-glass is then sealed to an underlying ground-glass plate by vaselin or paraffin, and the fluid is allowed to vaporize at ordinary room-temperature. The point here to be decided is the volume or weight of such a fluid that it is necessary to expose in an air-chamber of known cubic capacity in order that bacteria may be destroyed by its vapor in a given time.

In determining the germicidal value of different chemical agents for certain pathogenic bacteria susceptible animals are sometimes inoculated with the organisms after they have been exposed to the disinfectant. If no pathological condition results, disinfection is assumed to have been successful; while if the condition characteristic of the activities of the given organism in the tissues of this animal appears, the reverse is the case. The objections to this method are: "First. The test-organisms may be modified as regards reproductive activity without being killed; and in this case a modified form of the disease may result from the inoculation, of so mild a character as to escape observation.

Second. An animal that has suffered this modified form of the disease enjoys protection, more or less perfect, from future attacks, and if used for a subsequent experiment may, by its immunity from the effects of the pathogenic test-organism, give rise to the mistaken assumption that this had been destroyed by the action of the germicidal agent to which it had been subjected." (Sternberg.)

DETERMINATION OF ANTISEPTIC PROPERTIES.

For this purpose sterile media are employed, and are usually arranged in two groups: the one to remain normal in composition and to serve as controls, while to the other the substance to be tested is to be added in different but known strengths. It is customary to employ test-tubes each containing an exact amount of bouillon, gelatin, or agar-agar, as the case may be. To each tube a definite amount of the antiseptic is added, and if it is not of a volatile nature or not injured by heat, the tubes may then be sterilized. After this they are to be inoculated with the organism with which the test is to be made, and at the same time one of the "control"-tubes (one of those to which no antiseptic has been added) is inoculated. They are all then to be placed in the incubator and kept under observation. If at the end of twenty-four, forty-eight, or seventy-two hours no growth appears in any but the "control"-tubes, it is evident that the antiseptic must be added in smaller amounts, for we are to determine the point at which it is *not* as well as that at which it is capable of preventing development. The experiment is then repeated, using smaller amounts of the antiseptic until we reach a point at which growth just occurs, notwithstanding the presence

of the antiseptic; the amount necessary for antisepsis is then a trifle greater than that used in the last tube. If, for example, there was no development in the tubes in which the antiseptic was present in the proportion of 1:1000, and growth in the one in which it was present in 1:1400, the experiment should be repeated with strengths of the antiseptic corresponding to 1:1000, 1:1100, 1:1200, 1:1300, 1:1400, and in this way one ultimately determines the amount by which growth is *just* prevented; this represents the antiseptic value of the substance for the organism with which it was tested.

EXPERIMENTS.

To each of three tubes containing 10 c.c.—one of physiological salt-solution, another of bouillon, a third of fluid blood-serum—add as much of a culture of *micrococcus aureus* as can be held upon a looped platinum wire. Break this up carefully to eliminate clumps, and then add exactly 10 c.c. of a 1:500 solution of corrosive sublimate. Mix thoroughly, and at the end of three minutes transfer a drop from each tube into tubes of liquefied agar-agar, and pour these into Petri dishes. Label each dish carefully and place them in the incubator. Are the results the same in all the plates? How are the differences to be explained? To what strength of the disinfectant were the organisms exposed in the experiment?

To each of two tubes—the one containing 10 c.c. of physiological salt-solution, the other of bouillon—add as much of a spore-containing culture of anthrax bacilli as can be held upon a loop of platinum wire. Distribute this uniformly through the medium, and then add exactly 10 c.c. of a 1:500 solution of corrosive sublimate. Mix thoroughly,

and at the end of five minutes transfer a drop from each tube to tubes of liquefied agar-agar. Pour these immediately into Petri dishes. Label each dish carefully and place them in the incubator. Note the results at the end of twenty-four, forty-eight, and seventy-two hours. How do you explain them?

Make identically the same experiment with the same spore-containing culture of anthrax bacilli, except that the drop from the mixture is to be transferred to 10 c.c. of a mixture of equal parts of ammonium sulphide and sterilized distilled water. After remaining in this for about half a minute, a drop is to be transferred to a tube of liquefied agar-agar, poured into Petri dishes, labelled, and placed in the incubator. Note the results. Do they correspond with those obtained in the preceding experiment? How are the differences explained?

Prepare a 1:1000 solution of corrosive sublimate. To each of twelve tubes containing exactly 10 c.c. of bouillon add one drop to the first, two drops to the second, and so on until the last tube has had twelve drops added to it. Mix thoroughly and then inoculate each with one wire-loopful of a bouillon culture of *micrococcus aureus*. Place them all in the incubator after carefully labelling them. Note the order in which growth appears.

Do the same with anthrax spores, with spores of *bacillus subtilis*, and with the typhoid bacillus, and compare the results. From these experiments, what will be the strength of corrosive sublimate necessary to antisepsis under these conditions for the organisms employed?

Make a similar series of experiments using a 5 per cent. solution of carbolic acid.

Determine the antiseptic value of the common disinfectants for the organisms with which you are working.

Determine the time necessary for the destruction of the organisms with which you are working, by corrosive sublimate in 1:1000 solution, under different conditions—with and without the presence of albuminous bodies other than the bacteria, and under varying conditions of temperature.

In making these experiments be careful to guard against the introduction of sufficient sublimate into the agar-agar with which the Petri plate is to be made to inhibit the growth of the organisms which may not have been destroyed by the sublimate. This may be done by transferring two drops from the mixture of sublimate and organism into not less than 10 c.c. of sterilized physiological salt solution, in which they may be thoroughly shaken for from one to two minutes, or into the solution of ammonium sulphide of the strength given.

To 10 c.c. of a bouillon culture of *micrococcus aureus* or anthrax spores add 10 c.c. of a 1:500 solution of corrosive sublimate, and allow it to remain in contact with the organisms for *only one-half* the time necessary to destroy them (use an organism for which this has been determined). Then transfer a drop of the mixture to each of three liquefied agar-agar tubes and pour them into Petri dishes. Place them in the incubator and observe them for twenty-four, forty-eight, and seventy-two hours. No growth occurs. How is this to be accounted for?

At the end of seventy-two hours inoculate all of these plates with a culture of the same organism which has *not been exposed* to sublimate, by taking up bits of culture on

a needle and drawing it across the plates. A growth now results. We have here an experiment in which organisms which have been exposed to sublimate for a *much shorter time* than necessary to destroy them, when transferred directly to a favorable culture-medium do not grow, and yet, when the same organism which has not been exposed to sublimate at all is planted upon the same medium it does grow. How is this to be accounted for?

SKIN-DISINFECTION.

With a sterilized knife scrape from the skin of the hands, at the root of the nails, and under the nails, small particles of epidermis. Prepare plates from them. Note the results.

Wash the hands carefully for ten minutes in hot water and scrub them during this time with soap and a sterilized brush. Rinse them in hot water. Again prepare plates from scrapings of the skin on the fingers, at the root of the nails, and under the nails. Note the results.

Again wash as before in hot water with soap and brush, rinse in hot water, then soak the hands for five minutes in 1 : 1000 corrosive sublimate solution, and, as before, prepare plates from scrapings from the same localities. Note the results.

Repeat this latter procedure in exactly the same way, but before taking the scrapings let some one pour ammonium sulphide over the points from which the scrapings are to be made. After it has been on the hands about three minutes again scrape, and note the result upon plates made from the scrapings.

Wash as before in hot water and soap, rinse in clean hot water, immerse for a minute or two in alcohol, after this in

1 : 1000 sublimate solution, and finally in ammonium sulphide, and then prepare plates from scrapings from the points mentioned.

In what way do the results of these experiments differ from one another?

To what are these differences due?

What have these experiments taught?

In making the above experiments it must be remembered that the strictest care is necessary in order to prevent the access of germs from without into our media. The hand upon which the experiment is being performed must be held away from the body and must not touch any object not concerned in the experiment. The scraping should be done with the point of a knife that has been sterilized in a flame and allowed to cool. The scrapings may be transferred directly from the knife-point to the gelatin by means of a sterilized platinum wire loop.

The brush used should be thoroughly cleansed and always kept in 1 : 1000 solution of corrosive sublimate. It should be washed in hot water before using.

CHAPTER XIX.

Micrococcus Aureus—Micrococcus Pyogenes and Citreus—Staphylococcus Epidermidis Albus—Streptococcus Pyogenes—Micrococcus Gonorrhœæ—Micrococcus Intracellularis—Pseudomonas Æruginosa—Bacillus of Bubonic Plague.

MICROCOCCUS AUREUS (ROSENBACH), MIGULA, 1900.

SYNONYMS: *Staphylococcus pyogenes aureus*, Rosenbach, 1884; *Micrococcus pyogenes aureus*, Migula, 1895; *Micrococcus pyogenes*, Lehmann and Neumann, 1896.

PREPARE a set of plates of agar-agar from the pus of an acute abscess or boil that has been opened under antiseptic precautions. Care must be taken that none of the antiseptic used gains access to the culture-tubes, otherwise its restraining effect may be operative and the development of the organisms interfered with. It is best, therefore, to take a drop of the pus upon a platinum-wire loop after it has been flowing for a few seconds; even then it must be taken from the mouth of the incision and before it has run over the surface of the skin. At the same time prepare two or three coverslips from the pus.

Microscopic examination of these slips will reveal the presence of a large number of pus-cells, both multi-nucleated and with horseshoe-shaped nuclei, some threads of disintegrated and necrotic connective tissue, and, lying here and there throughout the preparation, small round bodies which will sometimes appear singly, sometimes in pairs, and frequently will be seen grouped together somewhat like clusters of grapes. (See Fig. 66.) They stain readily and

are commonly located in the material between the pus-cells; very rarely they may be seen in the protoplasmic body of the cell. (Compare the preparation with a similar one made from the pus of gonorrhea. (See Fig. 69.) In what way do the two preparations differ, the one from the other?

After twenty-four hours in the incubator the plates will be seen to be studded here and there with yellow or orange-colored colonies, which are usually round, moist, and glistening in their naked-eye appearances. When located in

FIG. 66



Preparation from pus, showing pus-cells, *A*, and micrococci, *C*.

the depths of the medium they are commonly seen to be lozenge or whetstone in shape, while often they appear as irregular stars with blunt points, and again as irregularly lobulated dense masses. In structure they are conspicuous for their density. Under the low objective they appear, when on the surface, as coarsely granular, irregularly round patches, with more or less ragged borders and a dark irregular central mass, which has somewhat the appearance of masses of coarser clumps of the same material as that com-

posing the rest of the colony. Microscopically, these colonies are composed of small round cells, irregularly grouped together. They are in every way of the same appearance as those seen upon the original cover-slip preparation.

Prepare from one of these colonies a pure stab-culture in gelatin. After thirty-six to forty-eight hours liquefaction of the gelatin along the track of the needle, most conspicuous at its upper end, will be observed. As growth continues the liquefied portion becomes more or less of a stocking-shape, and gradually widens at its upper end into an irregular funnel. This will continue until the whole of the gelatin in the tube eventually becomes fluid. There can always be noticed at the bottom of the liquefying portion an orange-colored or yellow mass composed of a number of the organisms which have sunk to the bottom of the fluid.

On potato the growth is quite luxuriant, appearing as a brilliant, orange-colored layer, somewhat lobulated and a little less moist than when growing upon agar-agar.

It does not produce fermentation with gas-production.

It belongs to the group of facultative anaërobes.

In milk it causes coagulation with acid reaction. This is, however, variable.

It is not motile, and being of the family of micrococci does not form endogenous spores. It possesses, however, a degree of resistance to detrimental agencies that is somewhat greater than that common to non-spore-bearing bacteria.

In bouillon it causes a diffuse clouding, and after a time a yellow or orange-colored sedimentation.

This organism is the commonest of the pathogenic bacteria with which we shall meet. It is *micrococcus aureus*, or as it is more commonly known, the *staphylococcus aureus*,

and is the organism most frequently concerned in the production of acute, circumscribed, suppurative inflammations. As it is almost ubiquitous, it is a source of continuous annoyance to the surgeon.

While it is the etiological factor in the production of most of the suppurative processes in man, still it is with no little difficulty that these conditions can be reproduced experimentally in lower animals. Its subcutaneous introduction into their tissues does not always result in abscess-formation, and when it does there is probably coincident interference with the circulation and nutrition of these tissues which renders them less able to resist its inroads. When introduced into the great serous cavities of the lower animals its presence is likewise not always accompanied by the production of inflammation. If the abdominal cavity of a dog, for example, be carefully opened so as to make as slight a wound as possible, and no injury be done to the intestines, large quantities of bouillon cultures or watery suspensions of this organism may be, and repeatedly have been introduced into the peritoneum without the slightest injury to the animal. On the contrary, if some substance which acts as a direct irritant to the intestines—such, for example, as a small bit of potato upon which the organisms are growing—be at the same time introduced, or the intestines be mechanically injured, so that there is a disturbance in their circulation, then the introduction of these organisms is promptly followed by acute and fatal peritonitis. (Halsted.¹)

On the other hand, the results which follow their introduction into the circulation are practically constant. If one

¹ The Johns Hopkins Hospital Reports. Report in Surgery, No. 1, 1891, ii, No. 5, 301-303.

inject into the circulation of the rabbit through a vein of the ear, or in any other way, from 0.1 to 0.3 c.c. of a bouillon culture or watery suspension of a virulent variety of this organism, a fatal pyemia always follows in from two and one-half to three days. A few hours before death the animal suffers frequently from severe convulsions. Now and then excessive secretion of urine is noticed. The animal may appear in moderately good condition until from eight to ten hours before death. At the autopsy a typical picture presents: the voluntary muscles are seen to be marked here and there by yellow spots, which average the size of a flaxseed, and are of about the same shape. They lie usually with their long axis running parallel to the muscle-fibres. As the abdominal and thoracic cavities are opened the diaphragm is often seen to be studded with them. Frequently the pericardial sac is distended with a clear gelatinous fluid, and almost constantly the yellow points are seen in the myocardium. The kidneys are rarely without them; here they appear on the surface as isolated yellow points, or, again, are seen as conglomerate masses of small yellow points which occupy, as a rule, the area fed by a single vessel. If one make a section into one of these yellow points, it will be seen to extend deep down through the substance of the kidney as a yellow, wedge-shaped mass, the base of the wedge being at the surface of the organ.

It is very rare that these abscesses—for abscesses the yellow points are, as we shall see when we come to study them more closely—are found either in the liver, spleen, or brain; their usual location being, as said, in the kidney, myocardium, and voluntary muscles.

These minute abscesses have a dry, cheesy, necrotic centre, in which the micrococci are present in large numbers

as may be seen upon cover-slips and in cultures prepared for them.

Preserve in alcohol bits of all tissues in which the abscesses are located. When these tissues are hard enough to cut sections should be made through the abscess-points and the histological changes carefully studied.

Microscopic Study of Cover-slips and Sections.—In cover-slip preparations this organism stains readily with the ordinary dyes. In tissues, however, it is best to employ some method by means of which contrast-stains may be utilized, and the location and grouping of the organisms in the tissues rendered more conspicuous. When stained, sections of tissues containing the small abscesses present the following appearances:

To the naked eye will be seen here and there in the section, if the abscesses are very numerous, small, darkly stained areas which range in size from that of a pin-point up to those having a diameter of from 1 to 2 mm. These points, when in the kidney, may be round or oval in outline; or may appear wedge-shaped, with the base of the wedge toward the surface of the organ. The differences in shape depend frequently upon the direction in which the section has been made through the kidney. In the muscles they are irregularly round or oval.

When quite small they appear, in stained sections, to the naked eye, as simple, round or oval, darkly stained points; but when they are in a more advanced stage a pale centre can usually be made out.

When magnified they appear in the earliest stages as minute aggregations of small cells, the nuclei of which stain intensely. Almost always evidences of progressing necrosis can be seen about the centre of these cell-accumulations.

The normal structure of the cells of the tissues is more or less destroyed; there is seen a granular condition due to cell-fragmentation; at different points about the centre of this area the tissue appears cloudy and the tissue-cells do not stain readily. Round about and through this spot are seen the nuclei of pus-cells, many of which are undergoing disintegration. In the smallest of these beginning abscesses the micrococci are to be seen scattered about the centre of the necrotic tissue; but in a more advanced stage they are commonly seen massed together in very large numbers in the form commonly referred to as *emboli of micrococci*, meaning, obviously, that they had developed within the lumen of a tiny bloodvessel.

When the process is well advanced, the different parts of the abscess are more easily detected. They then present in sections somewhat the following conditions: at the centre can be seen a dense, granular mass which stains readily with the basic aniline dyes, and when highly magnified is found to be made up of micrococci. Sometimes the shape of this mass of micrococci corresponds to that of the capillary in which the organisms became lodged and developed. Immediately about the embolus of cocci the tissues are in an advanced stage of necrosis. Their structure is almost completely destroyed, although the destruction is seen to be more advanced in some of the elements of the tissues than in others. As we approach the periphery of this faintly stained necrotic area it becomes marked here and there with granular bodies, irregular in size and shape, which stain in the same way as do the nuclei of the pus-cells and represent the result of disintegration going on in these cells.

Beyond this area we come upon a dense, deeply stained zone, consisting of closely packed pus-cells; of granular

detritus resulting from destructive processes acting upon these cells; and of the normal cellular and connective-tissue elements of the part. Here and there through this zone will be seen localized areas of beginning death of the tissues. This zone gradually fades away into the healthy surrounding tissues. It constitutes the so-called "abscess-wall."

Such is the picture presented by the miliary abscess when produced experimentally in the rabbit, and it corresponds from beginning to end with the pathological changes which accompany the formation of larger abscesses in the tissues of human beings.

From these small abscesses in the tissues of the rabbit *micrococcus aureus* may again be obtained in pure culture, and will present identically the same characteristics that were possessed by the culture with which the animal was inoculated.

A characteristic of all staphylococcus abscesses, small as well as large, is centralized death of tissue; even in those of microscopic dimensions this area of necrosis is always discernible by appropriate methods of examination. It represents the very starting-point of the destructive process, and is referable to the combined action of the endotoxins of the bacteria and the interference with the circulation of the part due to proliferation of cells about the point at which the bacteria are located.

As a result of the studies of van de Velde, Krauss, von Lingelsheim, Neisser and Wechsberg, and others, our knowledge of the poison that causes the destruction—staphylotoxin, as it is called—has been greatly extended. Through the work of these investigators we now know that the pathogenic properties of *micrococcus aureus* are due to a definite soluble toxin elaborated by it: that this poison is produced

under artificial conditions of cultivation, and may be separated from the living organisms by filtration; that when injected into the living animal body its effects upon the tissues are essentially reproductions of those accompanying the growth of the organism itself; that when this action is tested upon particular cells, such as erythrocytes and leukocytes, two distinct properties are exhibited, one a hemolytic, through which the red corpuscles are dissolved, the other a leucocidic, through which the white blood-corpuscles are destroyed; that the hemolytic and leucocidic properties are distinct from one another, and are due to the activities of two lysins, of which the staphylotoxin is (in part?) composed, and which may be separated from one another by appropriate methods of analysis; that the result of the treatment of animals with gradually increasing non-fatal doses of staphylotoxin is the appearance in the blood of the animals of antibodies (antilyns) that inhibit the action of the toxins (lysins); and, finally, that in the serum of certain animals (man and horse) similar antilyns in varying amounts are normally present.¹

Petersen, Paltchikowsky, Pröscher, and others have recently attempted to prepare an antistaphylococcus serum with the following results: The serum of patients recovering from severe staphylococcus infections contains protective substances which serve to protect rabbits from twice the fatal dose of a staphylococcus culture. Similarly the serum of immunized rabbits and goats, as shown by the experiments of Petersen, possesses about the same degree

¹ See van de Velde, *Annales de l'Institut Pasteur*, tome x, p. 580; Krauss *Wiener klin. Wochenschrift*, 1900, No. 3; Von Lingelsheim, *Etiologie und Therapie der Staphylokokken Infektion* (monograph), Berlin-Wien, 1900; Neisser and Wechsberg, *Zeitschrift für Hygiene und Infektionskrankheiten*, 1901, Bd. xxxvi, S. 299.

of protective powers. No antitoxic power could be demonstrated in the serum of the treated animals. The extremely limited degree of the protective power of antistaphylococcus serums makes them useless for curative purposes in human beings, as Petersen calculated that an adult would require from 350 to 700 c.c. of the serum at a single dose, as judged by its effects on the lower animals.

OTHER COMMON PYOGENIC ORGANISMS.

MICROCOCCUS PYOGENES (Rosenbach), Migula, 1900. Synonyms: *Staphylococcus pyogenes albus*, Rosenbach, 1884; *Micrococcus pyogenes albus*, Lehmann and Neumann, 1896.

MICROCOCCUS CITREUS (Passet), Migula, 1900. Synonym: *Staphylococcus pyogenes citreus*, Passet, 1895.

The pus of an acute abscess in the human being may sometimes contain organisms other than *micrococcus aureus*. *Micrococcus pyogenes* and *micrococcus citreus* may be found. The colonies of the former are white, those of the latter are lemon yellow. With these exceptions they are in all essential cultural peculiarities similar to *micrococcus aureus*. As a rule, they are not virulent for animals, and when they do possess pathogenic properties, it is in a much lower degree than is commonly the case with the golden micrococcus. *Streptococcus pyogenes* is also present sometimes. The commonest of the pyogenic organisms, however, is that just described, viz.: *micrococcus aureus*.

An organism that is almost universally present in the skin, and is often concerned in producing mild forms of inflammation, is *staphylococcus epidermidis albus* (Welch), an organism that readily may be confused with *micrococcus pyogenes*. It differs from the latter by the slowness with which it liquefies gelatin and by the comparative absence

of pathogenic properties when injected into the circulation of rabbits. Welsh regards this organism as a variety of *micrococcus pyogenes*.

**STREPTOCOCCUS PYOGENES (ROSENBACH), MIGULA,
1900.**

SYNONYMS: *Streptococcus*, Billroth, 1874; *Streptococcus pyogenes*, Rosenbach, 1884.

From a spreading phlegmonous inflammation prepare cover-slips and cultures. What is the predominating organism? Does it appear in the form of irregular clusters

FIG. 67

Streptococcus pyogenes in pus.

like those of grapes, or have its individuals a definite, regular arrangement? (See Fig. 67.) Are its colonies like those of *micrococcus aureus*?

Isolate this organism in pure cultures. In these cul-

tures it will be found on microscopic examination to present an arrangement somewhat like a chain of beads. (Fig. 68.)

Determine its peculiarities and describe them accurately. They should be as follows:

Upon microscopic examination a micrococcus should be found, but differing in its arrangement from those just described. The single cells are not scattered irregularly or arranged in clumps similar to bunches of grapes, but are joined together in chains like strands of beads. These strands are sometimes regular in the arrangement and size of the individual cells composing them, but more commonly

FIG. 68



Streptococcus pyogenes.

certain irregular groups may be seen in them. Here they appear as if two or three cells had fused together to form a link in the chain, so to speak, that is somewhat longer than the others; again, portions of the chain may be thinner than the rest, or it may appear broken or ragged. Commonly the individuals comprising this chain of cocci are not round, but appear flattened on the sides adjacent to one another. The chains are sometimes short, consisting of but four to six cells; or, again, they may be much longer, and extend from a half to two-thirds the way across the field of the microscope.

Under artificial conditions it sometimes grows well, and can be cultivated through many generations, while at other times it rapidly loses its vitality. When isolated from the diseased area upon artificial media it seems to retain its vitality for a longer period if replanted upon fresh media every day or two for a time; but if the first generation be transplanted and allowed to remain upon the original medium for from a week to ten days, it is not uncommon to find the organism incapable of further cultivation.

Under no conditions is its growth very luxuriant.

On gelatin plates its colonies appear after forty-eight to seventy-two hours as very small, flat, round points of a bluish-white or opalescent appearance. They do not cause liquefaction of the gelatin, and in size they rarely exceed 0.6–0.8 mm. in diameter. Under low magnifying power they have a brownish or yellowish tinge by transmitted light, and are finely granular. As the colonies become older their regular borders may become slightly irregular or notched.

In stab-cultures in gelatin they grow along the entire needle-track as a finely granular line, the granules representing minute colonies of the organism. On the surface the growth does not usually extend beyond the point of puncture.

On agar-agar plates the colonies appear as minute pearly points, which when slightly magnified are seen to be finely granular, of a light-brownish color, and regular at their margins.

When smeared upon the surface of agar-agar or gelatin slants the growth that results is a thin, pearly, finely granular layer, consisting of minute colonies growing closely side by side. Its most luxuriant growth is seen on glycerin-agar-

agar at the temperature of the body (37.5° C.), and its least on gelatin at from 18° to 20° C.

On blood-serum its colonies present little that is characteristic; they appear as small, moist, whitish points, from 0.6 to 0.8 mm. in diameter, that are slightly elevated above the surface of the serum. They do not coalesce to form a layer over the surface, but remain as isolated colonies.

On potato no visible development appears, but after a short time (thirty-six to seventy-two hours) there is a slight increase of moisture about the point of inoculation, and microscopic examination shows that multiplication of the organisms placed at this point has occurred.

In milk its conduct is not always the same, some cultures causing a separation of the milk into a firm clot and colorless whey, while others do not produce this coagulation. The latter, when cultivated in milk of a neutral or slightly alkaline reaction, to which a few drops of litmus tincture have been added, produce, as a rule, only a very faint pink color after twenty-four hours at 37° C.

In bouillon it grows as tangled masses or clumps, which upon microscopic examination are seen to consist of long chains of cocci twisted or matted together.

It grows best at the temperature of the body (37.5° C.), though development does occur at the ordinary room-temperature.

It is a facultative anaërobe.

It stains with the ordinary aniline dyes, and is not decolorized when subjected to Gram's method.

It is not motile. Under artificial conditions we have no reason to believe that it enters a stage in which its resistance to detrimental agencies is increased. In the tissues of the body, however, it appears to possess marked vitality,

for it is not rare to observe recurrences of inflammatory conditions due to this organism, often at a relatively long time after the primary site of infection has healed.

Streptococcus pyogenes is the organism most commonly found in rapidly spreading suppurations, while *micrococcus aureus* is most frequently found in circumscribed abscess formations; they may also be found together, and these relationships may be reversed at times.

The results of its inoculation into the tissues of lower animals are described by Rosenbach and Passet as protracted, progressive, erysipelatoid inflammations; and Fehleisen, who described a streptococcus in erysipelas that is in all probability identical with the streptococcus pyogenes under consideration, stated that it produced in the tissues of rabbits (the base of the ear) a sharply defined, migratory reddening without pus-formation. The writer encountered a strain of this organism that possessed the property of inducing erysipelas when introduced into the skin of the ear, and disseminated abscess-formation when injected into the circulation of rabbits. This observation has an important bearing upon the question concerning the identity of streptococci found in various inflammatory conditions, such, for instance, as the spreading erysipelatoid manifestations on the one hand, and the circumscribed abscess-formations on the other.

The results that follow upon the inoculation of animals with cultures of streptococci obtained from various inflammatory lesions are, as a rule, inconstant. At times cultures will be encountered that are apparently without virulence, no matter how tested; while again cultures from other sources exhibit the most marked pathogenic properties, even when employed in almost infinitesimal quantities.

Between these extremes every gradation may be expected. The virulence of a culture as exhibited upon animals under experiment is not necessarily proportional to the intensity of the pathological process from which it was derived.

There is never any certainty of faithfully reproducing, by inoculation into susceptible animals, the pathological lesion from which a culture of the organism may have been obtained. The introduction into a susceptible animal of a culture derived from either a spreading phlegmon or an erysipelatous inflammation may result in erysipelas, general septicemia, local abscess-formation, or, as said, may have no effect at all. Cultures may be encountered that are pathogenic for one susceptible species of animals and not for another.

Under the ordinary conditions of artificial cultivation fully virulent varieties of streptococcus pyogenes usually lose their virulence after a short time. Petruschky¹ preserves this property by cultivation upon nutrient gelatin for two days at 22° C., keeping the cultures after this time in the refrigerator, and transplanting upon fresh gelatin every five or six days. Marmorek² finds the virulence preserved by growing the organism in a mixture of 2 parts of horse or human blood-serum and 1 part of nutrient bouillon, or of 1 part of ascites-fluid and 2 parts of bouillon.

Its virulence may sometimes be increased by passage through a series of susceptible animals.

Certain authors are of the opinion that a relation exists between virulence and the length of the chains formed by streptococci when growing in fluid media. It is held that

¹ Centralblatt für Bakteriologie und Parasitenkunde, 1895, Abth. i, Bd. xvii.

² Annales de l'Institut Pasteur, 1895.

those forming the long chains, *streptococcus longus*, are the only ones concerned in animal pathology, and hence the only ones by which pathogenic powers may be exhibited; while those forming the short chains, *streptococcus brevis*, are not, as a rule, pathogenic, and may often be readily differentiated from the other variety by more or less gross cultural characteristics, such as slow liquefaction of gelatin, visible growth on potato, etc.¹

Antistreptococcus Serum.—Numerous investigators have demonstrated that certain animals—notably horses and asses—as well as some smaller animals, may be rendered immune from streptococcus pyogenes. Further, that in varying degrees the blood serum of such immune animals has both a curative and a prophylactic influence upon the course of streptococcus infection in human beings.

The method of producing the serum is, in general, to inject gradually increasing doses of virulent streptococcus pyogenes into the tissues of the animal until its blood serum is found to have an inhibiting effect upon experimentally produced streptococcus infection in test animals.

Reports upon the therapeutic use of antistreptococcus serum in a variety of streptococcus infections are discordant; some authors being enthusiastic as to its curative value, others skeptical or actually denying to it such virtues. The reasons for these divergent opinions are now pretty manifest. It seems well established, from studies of this and other infective organisms, that there are regularly encountered in various pathological processes different strains of one and the same species; strains that, while

¹ V. Lingelsheim, Zeitschrift für Hygiene, 1891, Band x, and 1892, Band xii; Behring, Centralblatt für Bakteriologie und Parasitenkunde, 1892, Band xii.

possessing all the common characteristics of the type species, reveal greater or less individual modification in some one or another functional particular. When such modification occurs in pathogenic power, or in some other essential function, as is often the case, it is probable that they may have an effect upon the results of immunization. At all events experience with the serum of animals immunized from closely allied though not identical varieties of a species obtained from various lesions, warrant this opinion. For instance: given *Streptococcus* A, B and C, obtained from different sources, there is no assurance that the serum of an animal immunized from strain A will necessarily act favorably in an infection caused by B or C, even though its action in infection caused by A may be entirely satisfactory, and *vice versa*. This experiment has led to the general conclusion that for favorable therapeutic action on the part of an antiserum it is advisable that such serum be obtained from animals immunized from the particular strain of infective organism that is concerned in the disease for which the serum is to be used in treatment.

This does not necessarily imply that the particular culture obtained from each of the manifold pathological lesions shall be used as the immunizing agent in efforts to secure an antiserum for the pathological condition from which it was derived, but rather suggests the desirability of establishing, through culture or other tests, sub-groups having more or less common peculiarities so that even though the members of group A may differ slightly in details from one another, such difference is nevertheless much less than that observed between themselves and the members of group B or C, and an antiserum produced through the use of either of the members of group A will act favorably upon

the infections caused by the members of group A, while it may be inactive upon those caused by the members of group B or C.

The method adopted by Cole in his work upon the treatment of pneumonia with antipneumococcus serum (to be described later) is essentially along this line and his results thus far point directly to the logic of the procedure.

NOTE.—If the opportunity presents, obtain cultures from a case of erysipelas. Compare the organism thus obtained with streptococcus pyogenes. Inoculate rabbits both subcutaneously and intravenously with about 0.2 c.c. of pure cultures of these organisms in bouillon. Do the results correspond, and do they in any way suggest the results obtained with micrococcus aureus when introduced into animals in the same way? Do these streptococci flourish readily on ordinary media?

THE LESS COMMON PYOGENIC ORGANISMS.

The organisms that have just been described are commonly known as the "pyogenic cocci" of Ogston, Rosenbach, and Passet, and up to as late as 1885 were believed to be the specific factors concerned in the production of suppurative inflammations. Since that time, however, there has been considerable modification of this view, and while they are still known to be the most common causes of suppuration, they are also known to be not the only causes of this process.

With the more general application of bacteriological methods to the study of the manifold conditions coming under the eye of the physician, the surgeon, and the pathologist, observations are constantly being made that do not

accord with the earlier ideas upon the dependence of all forms of suppuration on invasion by the pyogenic cocci. There is an abundance of evidence to justify the opinion that a number of organisms not commonly classed as pyogenic may, under certain circumstances, assume this property or may, in fact, have pus formation as one of the common accompaniments of their pathogenic activities. For example:

The bacillus of typhoid fever has been found in pure culture in osteomyelitis of the ribs, in acute purulent otitis media, in abscess of the soft parts, in the pus of empyema, and in localized fibrino-peritonitis, either during its course or as a sequel of typhoid fever.

Bacillus coli communis has been found in pure culture in acute peritonitis, in liver-abscess, in purulent inflammation of the gall-bladder and ducts, and in appendicitis. Welch¹ found it in pure culture in fifteen different inflammatory conditions.

Micrococcus lanceolatus (pneumococcus) has been found alone in abscess of the soft parts, in purulent infiltration of the tissues about a fracture, in purulent cerebrospinal meningitis, in suppurative synovitis, in acute pericarditis, and in acute inflammation of the middle ear.

Organisms simulating *bacterium diphtheriticum* are frequently encountered in large numbers in the pus of superficial wounds, and especially in ulcerations of the skin and mucous membranes.

Moreover, many of the less common organisms have been detected in pure cultures in inflammatory conditions with which they were not previously thought to be concerned, and to which they are not usually related etiologically.

¹ Conditions Underlying the Infection of Wounds, American Journal of the Medical Sciences, November, 1891.

In consideration of such evidence as this it is plain that we can no longer adhere rigidly to the opinions formerly held upon the etiology of suppuration, but must subject them to modifications in conformity with this newer evidence. We now know that there exist bacteria other than the "pyogenic cocci," which, though not normally pyogenic, may give rise to tissue-changes indistinguishable from those produced by the ordinary pus-organisms.

Furthermore—of organisms not classified as of the "pyogenic group," but where growth in the tissues is always accompanied by pus formation—one may mention micrococcus gonorrhea, micrococcus intracellularis, and bacillus pestis.

MICROCOCCUS GONORRHEÆ (NEISSER), 1879.

SYNONYM: Gonococcus Neisser, Bumm, 1887.

One observes upon microscopic examination of cover-slips prepared from the pus of acute gonorrhea that many of the pus-cells contain within their protoplasm numerous small, stained bodies that are usually arranged in pairs. Occasionally a cell is seen that contains only one or two pairs of such bodies; again, a cell will be encountered that is packed with them. Occasionally masses of these small bodies will be seen lying free in the pus. (See Fig. 69.) The majority of the pus-cells do not contain them.

These small, round, or oval bodies are the so-called "gonococci" discovered by Neisser, and more fully studied subsequently by Bumm, to whom we are indebted for much of our knowledge concerning them.

As the name implies, this organism is a micrococcus, and as it is commonly arranged in pairs (flattened at the sur-

faces in juxtaposition) it is often designated as diplococcus of gonorrhea. It is always to be found in gonorrheal pus, and often persists in the genital discharges and secretions far into the stage of convalescence. It is not present in inflammatory conditions other than those of gonorrheal origin.

It is easily detected microscopically in the secretions of acute gonorrhea. In secondary lesions and in very old, chronic cases it is difficult of detection and frequently

FIG. 69



Pus of gonorrhea, showing diplococci in the bodies of the pus-cells.

eludes all efforts to find it. It is stained by the ordinary methods, but perhaps most satisfactorily with the alkaline solution of methylene-blue. Most important as a differential test is its *failure* to stain by the method of Gram. (How does this compare with the behavior of the other pyogenic cocci when treated in the same way?)

It does not grow upon ordinary nutrient media, and has only been isolated in culture through the employment of special methods. Its growth under artificial conditions seems to depend upon some particular nutrient substance that is supplied by blood or blood-serum, and in all the media

that have been successfully used for its cultivation this substance is apparently an essential constituent. By many investigators it is thought that only *human* blood possesses this important ingredient, though this opinion is not universal.¹

It was first isolated in culture by Bumm, who used for this purpose coagulated human blood-serum obtained from the placenta.

Wertheim improved the method of Bumm by using a mixture of equal parts of sterile human blood-serum and ordinary sterilized nutrient agar-agar, the latter having been liquefied and kept at 50° C. until after the mixture was made, when it was allowed to cool and solidify.

Other investigators have substituted for human blood-serum certain pathological fluids from the human body, such as ascites-fluid, fluid from ovarian cysts, and serous effusions from the pleura and from the joint-cavities.

The method used by Pfeiffer for the cultivation of *bacterium influenzae* (see that method) is also said to have been successfully employed.

Vedder's Medium.—A simple medium that has given satisfactory results in our hands is that devised by Vedder. It consists of ordinary beef infusion agar (1.5 per cent. agar) to which 1 per cent. of corn starch is added. The medium contains neither sodium chloride nor peptone and has a reaction corresponding to 0.2 to 0.5 per cent. acid to phenolphthalein.

Wright's modification of Steinschneider's method has given such satisfactory results in his hands that it will be given here somewhat in detail. The medium consists of a mixture of urine, blood-serum (human or bovine, either

¹ An instructive article on this subject is that by Foulerton, On *Micrococcus Gonorrhoeæ* and Gonorrheal Infection, Transactions of the British Institute of Preventive Medicine, 1897, series i.

serving the purpose), and nutrient agar-agar. The urine and blood-serum are collected without special aseptic precautions, and subsequently freed from bacteria by filtration through unglazed porcelain. Frequently this is the tedious part of the process, as the serum and urine pass very slowly through the porcelain filters generally employed in laboratories. Wright recommends a filtering cylinder manufactured by the Boston Filter Company as an apparatus that not only gives a sterile filtrate, but also permits of very rapid passage of the fluid.

The details of the method as given by Wright are as follows: "A liter of nutrient agar is prepared in the usual manner, and after filtration it is evaporated to about 600 c.c. This concentration is desirable, so that after dilution with the urine and serum the medium may be sufficiently firm. This concentrated agar is then run into test-tubes and the whole sterilized by steam. The quantity of agar placed in each tube is smaller than is usual; this is in order to allow for the subsequent addition of the urine and serum.

"The blood-serum, which need not be free from corpuscles, is first passed through white sand, which is supported in a funnel by filter-paper, in order to remove as far as is possible any particles in suspension, and is then mixed with half its volume of fresh urine. The mixture of urine and blood-serum is next filtered by suction through an unglazed porcelain cylinder into a receiving-flask, such as chemists use for similar purposes, by means of a water-vacuum pump. This frees the mixture from bacteria.

"The usual precautions are, of course, taken to prevent the contamination of the filtrate, such as the previous sterilization by steam of the cylinder and receiving-flask, besides others which will occur to any bacteriologist.

“To the agar in each test-tube, which is fluid and of a temperature of about 40° C., there is added about one-third to one-half its volume of the filtered mixture of urine and blood-serum. This is conveniently accomplished by pouring the mixture from the receiving-flask through the lateral tube, inserted near its neck directly into the tubes. The preliminary melting of the agar is best effected in the steam sterilizer, in order that any organisms which have found lodgment in the cotton plugs of the tubes may be destroyed. When the agar is melted it is cooled and kept fluid by placing the tubes in a water-bath at 40° C. Each tube, after the addition of the urine and serum to the fluid agar, is quickly shaken to insure a uniform mixture, and is then placed in an inclined position to allow the agar to solidify with a slanting surface. When the medium in the tubes has solidified the tubes are placed in the incubator for about twenty-four hours to test for contaminations, after which they are ready for use.”

The successive dilutions are now to be made upon the slanting surface of this mixture, as the mass in the tubes cannot be redissolved without exposure to a degree of heat that apparently interferes with the nutritive value of the serum contained in the medium.

When inoculated with gonorrheal pus, by smearing a loopful over the surface, the tubes are to be kept at from 37° to 38° C. The organism does not develop properly at a temperature below this point.

After twenty-four hours the colonies of the gonococcus appear on the surface of the medium, according to Wright, as very tiny, grayish, semitranslucent points. After forty-eight hours they may be about 1 millimeter or so in diameter, slightly elevated, with a rounded outline, grayish in color,

and semitranslucent by transmitted light. By reflected light their surface has the appearance of frosted glass. Later, if few in number, so that their growth is unimpeded, the colonies may attain a diameter of 2 millimeters or more, become thicker and denser, with a faintly brownish tinge about their centres, and a slightly irregular outline.

Under a low power of the microscope a fully developed colony is seen to consist of a general circular expansion, with thin, translucent, smooth, sharply defined margin, but becoming brownish, granular, and thicker toward the central portion, which is made up of coarse, granular, brown-colored clumps closely packed together.

The appearances coincide with the figure of such a colony given by Wertheim.¹

Wassermann² calls attention to the success he has had in cultivating this organism upon a mixture of swine-serum and nitrose, the latter being a commercial product chemically known as casein-sodium phosphate.

The preparation of the medium and its composition are as follows:

In an Erlenmeyer flask mix 15 c.c. of swine-serum, as free as possible from hemoglobin; 30 to 35 c.c. of water; 2 to 3 c.c. of glycerin; and finally 0.8 to 0.9 gram (*i. e.*, about 2 per cent.) of nitrose. This is boiled, with gentle agitation, over a free flame, until all ingredients are dissolved and the cloudy fluid has become quite clear. After such boiling the mixture can be sterilized by steam without precipitating the albumen, and may then be kept indefinitely ready for use.

¹ Deutsche med. Wochenschrift, 1891, No. 50; Centralblatt für Gynäkologie, 1891, No. 24.

² Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xvii, p. 298.

When needed, the flask and its contents are heated to 50° C.; from six to eight tubes of 2 per cent. peptone-agar-agar are dissolved by boiling, brought to 50° C., and then mixed with the solution in the flask and the mass poured into Petri dishes. Upon the surface of this serum-nitrose-agar the cultivation is to be conducted. Wassermann lays particular stress upon two points that are essential to success, viz., the preliminary boiling of the serum-nitrose mixture *before* steam sterilization, as this prevents precipitation of the albumin; and the necessity of having both the serum-nitrose mixture and the agar-agar, to be mixed with it, at not over 50° C., for if they are at a boiling temperature when mixed, or if they are brought to the boiling temperature *after* mixing, the albumin will be precipitated notwithstanding the presence of the nitrose, which otherwise prevents this.

Wassermann further observes that some samples of serum require to be more highly diluted with water than in the proportions given above; that the agar-agar should be feebly, but distinctly, alkaline to litmus, causing no red-denning whatever of blue litmus paper; and, finally, that the Petri dishes containing the solidified medium on which the cultures are growing are best kept bottom upward, so as to prevent water of condensation collecting on the surface. By the use of the above medium he has cultivated the gonococcus from about one hundred different cases.

Lipschütz's Medium.—Lipschütz¹ endeavored to find a medium that could be prepared easily from substances occurring in commerce. After testing a number of albuminous preparations of vegetable and animal origin, he

¹ Centralblatt für Bacteriologie, Originale, Bd. xxxvi, 1904.

selected the pulverized egg-albumen of Merck for this purpose. The culture-medium is prepared as follows: A 2 per cent. solution of the egg-albumen is made in water, to which is added 20 c.c. of a tenth-normal caustic soda solution per 100 c.c. of fluid, and this is allowed to stand for one-half hour, being agitated from time to time. It is then filtered and placed in Erlenmeyer flasks in amounts of 30 to 50 c.c., and sterilized by the intermittent method. The medium, when thus prepared, is colorless, transparent, of a light-yellow color, and reacts distinctly alkaline to litmus-paper. To this medium nutrient agar-agar or the ordinary bouillon may be added in the proportion of one part of the egg-albumen medium to two or three parts of the agar medium or the bouillon, and this he calls the "egg-albumen-agar" or the "egg-albumen-bouillon medium, on which *micrococcus gonorrhœæ* grows very satisfactorily. The special advantages claimed for this medium are that it can be prepared at any time and without difficulty, is quite clear and transparent, and permits, where agar-agar is used, the employment of the medium for the study of colony formations.

If *micrococcus gonorrhœæ* be transplanted from the original culture to either glycerin-agar-agar or to Löffler's serum-mixture, a growth is sometimes observed, more often in the latter than in the former, but of so feeble a nature that these substances cannot be regarded as suitable for its cultivation and certainly not for its direct isolation from the body. As a rule, development does not occur on glycerin-agar.

Microscopic examination of colonies of this organism reveals the presence of a diplococcus somewhat larger than the ordinary pyogenic cocci. The opposed surfaces of the

individual cells that comprise the couplets are flattened and separated by a narrow slit. At times the cocci are arranged as tetrads.

This organism cannot be grown at a temperature lower than that of the human body, and cultures that have been obtained by either of the favorable methods are said to lose their vitality when kept at ordinary room-temperature for about two days.

It is killed in a few hours by drying.

Cultures retain their vitality under favorable conditions of nutrition, temperature, and moisture for from three to four weeks.

This organism is without pathogenic properties for monkeys, dogs, and horses, as well as for the ordinary smaller animals used for this purpose in the laboratory.

In man typical gonorrhea has been produced on several occasions by the introduction into the urethra of pure cultures of this organism.

In addition to its causal relation to specific urethritis, it is the cause of gonorrheal prostatitis in man, of gonorrheal proctitis in both sexes, and of gonorrheal inflammation of the urethra, of Bartholin's glands, of the cervix uteri, and of the vagina in women and young girls. It is etiologically related to the specific conjunctivitis (ophthalmia neonatorum) of young infants, and also occasionally to ophthalmia in adults.

Secondarily, it is concerned in specific inflammations of the tubes and ovaries, of the lymphatics communicating with the genitalia, of the serous surfaces of joints, and of those of the heart, lungs, and abdominal cavity.

Other species of micrococci have from time to time been

described as occurring in the pus of acute urethritis and of other purulent inflammations. Many of these are of no significance. Some of them possess peculiarities that might lead to confusion. The diplococcus described by Heiman¹ has certain points of resemblance to the gonococcus, such as its location in the bodies of pus-cells, its grouping as diplococci, its size and general appearance; but it is still readily distinguished from the gonococcus by its retention of color when treated by Gram's method. The diplococcus detected by Bumm in puerperal cystitis is likewise often found within pus-cells, but it is readily differentiated from the gonococcus by its growth upon ordinary nutrient media. *Micrococcus intracellularis* of Weichselbaum, isolated from the sero-purulent fluid of the spinal canal in cases of epidemic cerebrospinal meningitis, is microscopically also strikingly like the gonococcus as it is seen in pus; but, unlike the latter organism, may be cultivated by the ordinary methods.

Micrococcus catarrhalis, so often seen within the bodies of pus cells in the nasal discharges of acute catarrh also suggests the organism under consideration, but is easily differentiated by its growth on the ordinary culture media.

Summary of Distinguishing Peculiarities.—Since gonorrheal discharges may be contaminated with pyogenic cocci other than those causing the specific inflammation, it is important in efforts to identify the gonococcus that the differential tests be borne in mind and put into practice. The gonococcus is differentiated from the commoner pyogenic organisms by the following peculiarities.

First, it is practically always seen in the form of diplococci,

¹ New York Medical Record, June 22, 1895.

the pair of individual cells having the appearance of two hemispheres, with the diameters opposed, and separated from one another by a narrow, colorless slit. (Is this the case with micrococcus aureus or streptococcus pyogenes?)

Second, in gonorrheal pus it is nearly always *within* the protoplasmic bodies of pus-cells. (How does this compare with the conditions found in ordinary pus?)

Third, it stains readily with the ordinary staining-reagents, *but loses its color when treated by the method of Gram*. (Treat a cover-slip from ordinary pus by this method and note the result.)

Fourth, it does not develop upon any of the ordinary media used in the laboratory; while the common pus-organisms, with perhaps the exception of the streptococci, are vigorous growers and are not markedly fastidious as to their nutritive medium.

Fifth, when obtained in pure culture by either of the special procedures noted above, its cultivation may be continued upon the same medium; but growth will usually not be observed if it is transplanted to ordinary nutrient gelatin, agar-agar, bouillon, or potato; should it grow under these circumstances its development will be very feeble. (Is this the case with common pus-producers?)

Sixth, it has no pathogenic properties for animals, while several of the pyogenic cocci, notably micrococcus aureus and streptococcus pyogenes, are usually capable of exciting pathological conditions. (This is less commonly true of streptococcus pyogenes than of micrococcus aureus.)

**MICROCOCCUS INTRACELLULARIS (WEICHSELBAUM),
MIGULA, 1900.**

SYNONYMS: *Diplococcus Intracellularis Meningitidis*, Weichselbaum, 1887; *Streptococcus Intracellularis* (Weichselbaum), Lehmann and Neumann, 1896.

Of the several organisms mentioned that might be mistaken for the gonococcus, no one of them is as suggestive and none, *per se*, so important as that concerned in the causation of epidemic cerebrospinal meningitis.

This organism, described by Weichselbaum in 1887 under the name "*diplococcus intracellularis meningitidis*," was found by him in the exudations of the brain and spinal cord in six cases of acute cerebrospinal meningitis.

As its name implies, it is a diplococcus, practically always seen within the bodies of pus-cells (polymorphonuclear leukocytes) in the exudations characteristic of this disease. It is not seen within the other cells of the morbid process. It stains readily with any of the ordinary aniline dyes, but is decolorized by the method of Gram. It is conspicuous for the irregular way in which it takes up the dye, some cells in a preparation (either from the exudate or from cultures) being brightly and intensely colored, others being much less so, or, indeed, often nearly colorless. There is also a marked variation in the size of individual cocci, some being normal, others being apparently swollen. These latter are often pale, with a deeply staining centre, giving the appearance of a coccus surrounded by a capsule; it is not improbable that these are degenerated. The irregularities here noted are more common in cultures than in fresh exudates from acute cases, and more common in old than in young cultures, a state of affairs fully explained

by the self-digestion (autolysis) that this organism is known to experience under conditions of artificial cultivation.

As seen in cultures, it is commonly arranged in pairs with the individuals flattened at the surfaces of juxtaposition. Sometimes it is seen grouped as four and occasionally as short chains of three or four cells, but never as long chains. Its size is that of the common pyogenic micrococci, and its outline and arrangement in the pus-cells are so like those of the gonococcus that the figure depicting gonorrheal pus answers equally well to illustrate the appearance of the exudate from acute meningitis.

Though facultative, still its parasitic nature is so dominant that it can only be cultivated with difficulty and uncertainty. The most satisfactory medium for its isolation in pure culture from the diseased meninges is coagulated blood-serum (Löffler's mixture), and even here one is not successful with each attempt. So uncertain is its growth under artificial conditions that it is always advisable to inoculate a number of tubes with relatively large quantities of the exudate, and even then growth often occurs in only a part of them, notwithstanding the fact that on microscopic examination the organism may have been readily detected in large numbers in the exudate. Illustrative of this difficulty, the following experience of Councilman, Mallory, and Wright may properly be quoted:¹

"As showing the difficulty in growing the organisms in cultures made from the meninges at the postmortem examination, ten cultures were made in one case from the exudation on the brain and six from the cord, cover-slip examinations showing abundant organisms in the cells. Only

¹ See Epidemic Cerebrospinal Meningitis, etc., Report of the State Board of Health, Mass., 1898, by Councilman, Mallory, and Wright.

two of the cultures from the brain and one from the cord showed a growth. As a rule, the organisms were more easily obtained in cultures made from the acute cases than from the chronic."

When successfully isolated in pure culture its growth is never profuse on any medium. On the serum mixture of Löffler the isolated colonies appear as round, viscid, smooth, sharply defined points that may attain a diameter of 1 to 1.5 mm. There is no liquefaction of the medium. Cultures from very acute cases occasionally present an abundant growth of fine, transparent colonies strongly suggestive of those of *micrococcus lanceolatus*.

On glycerin-agar the colonies are round, pearly, translucent, flat, and viscid in appearance. They tend to become confluent. Under low magnifying power they are homogeneous, semitransparent, faintly brownish, with well-defined smooth margins. On plain agar the growth is feeble and uncertain.

Its growth in bouillon is slow and uncertain. It does not cause clouding of the fluid, but collects at the bottom of the tube as a scanty grayish sediment, that when disturbed gives the impression of having a mucoid consistency.

It does not grow on potato and causes no change in litmus-milk.

It grows only at the temperature of the body, and can be kept growing only by being transplanted to fresh media about every two days, and even then growth often ceases after a comparatively small number of transplantations. If from a fresh growing culture a number of tubes be inoculated and kept under favorable conditions, it is a common experience to have growth on only a part of them. It is sometimes impossible to obtain a second growth on agar-agar.

In addition to its presence in the meningeal exudation of epidemic cerebrospinal meningitis, this organism may appear as a secondary invader of the lung, causing more or less extensive pneumonic exudation; of the joints; the ear; the eye; and the nose and throat. Though rarely, its presence in the circulating blood may sometimes be demonstrated (Gwynn).

Subcutaneous inoculation with pure cultures has usually no effect. Injections into the great serous cavities may or may not result in serofibrinous or fibrinopurulent inflammation. Positive results are oftener obtained on young guinea-pigs weighing about 150 grams, than on larger, more mature animals. Intravenous inoculations are equally unsatisfactory, though the results depend upon the original virulence, the age of the culture and the animal selected. In horses toxic symptoms are often the conspicuous result of this mode of inoculation.

The only successful attempts to reproduce the morbid conditions from which the organism is obtained are those in which the living cultures have been injected directly into the meninges. Weichselbaum produced congestion with pus formation in the meninges of dogs and rabbits by direct injection through openings made in the skulls; Councilman, Mallory, and Wright caused the death of a goat by the injection into the spinal canal of 1 c.c. of a bouillon suspension of a pure culture of the organism, the autopsy revealing intense congestion of the meninges of both brain and cord, with slight clouding of the meninges and slight increase of meningeal fluid, and Flexner¹ succeeded, through injections of cultures into the spinal canal of monkeys, in

¹ Jour. Exp. Med., 1907, ix, 168.

causing death of the animals with inflammation of the meninges of the cord and brain.

While the portal of entry for this organism to the system is not known, it is still of importance to note that it often makes its exit from the body by way of the organs that are secondarily involved and that open to without, as the ear, nose, eye, and lungs.

It is of equal importance to note that the organism is of very low power of resistance, being destroyed in twenty-four hours by direct sunlight and by drying at body-temperature, and in seventy-two hours by drying in the dark at ordinary room-temperature.

For the diagnosis of epidemic cerebrospinal meningitis by bacteriological methods it is essential that the meningeal fluid be obtained by lumbar puncture during the most acute stage of the disease.

ANTIMENINGITIS SERUM.¹ Flexner has demonstrated that the blood serum of horses and of goats that have received repeated subcutaneous injections of cultures of *diplococcus meningitidis* possesses a marked restraining action upon the course of meningitis. This is true not only for the experimental manifestations of the disease, but for those occurring in man as well. The analysis of about 400 cases of true epidemic cerebrospinal meningitis in man in which the serum was used shows that the general death rate was considerably lower than that following any other known mode of treatment. For cases treated between the first and third days of the disease it was as low as 16.5 per cent., while for those treated as late as, and later than the seventh day, it was 35 per cent. Between these figures

¹ Flexner and Jobling, *Arch. of Pediatrics*, 1908, p. 747.

the rates ran from 20 to 25 per cent. For success, therefore, early diagnosis and early administrations of the serum are essential.

**PSEUDOMONAS ÆRUGINOSA (SCHRÖTER, 1872),
MIGULA, 1900.**

SYNONYMS: *Bacterium Æruginosum*, Schröter, 1872; *Bacillus Æruginosus*, Schröter, 1872; *Bacillus Pyocyaneus*, Gessard, 1882; *Pseudomonas Pyocyanea*, Migula, 1896.

Another common organism that may properly be mentioned at this place, though perhaps not strictly pyogenic, is a pseudomonas frequently found in discharges from wounds, viz., *pseudomonas æruginosa*, or *bacillus pyocyaneus* or "bacillus of green pus," or of blue pus, or of blue-green pus, as it is by custom variously designated. *Pseudomonas æruginosa* is a delicate rod with rounded or pointed ends. It is actively motile; does not form spores. As seen in preparations made from cultures, it is commonly clustered in irregular masses. It does not form long filaments, there being rarely more than four joined end to end, and most frequently occurs as single cells.

It grows readily on all artificial media, and gives to some of them a bright-green color that is most conspicuous where it is in contact with the air. This green color, which becomes more and more marked as growth advances, is not seen in the growth itself to any extent, but is diffused through the medium on which the organism is developing. Ultimately this color becomes much darker, and in very old cultures may become almost black (sometimes very dark blue-green, at others brownish-black, at others more or less of a claret red).

NOTE.—To a fresh agar culture of this organism, in which the green coloration of the medium is especially marked, add about 2 c.c. of chloroform. Shake gently, and note that the chloroform extracts a blue coloring-matter from the culture, leaving the latter more or less yellow.

FIG. 70

FIG. 71



FIG. 72



FIG. 70.—Stab-culture of *ps. aeruginosa* in gelatin after twenty-eight hours at 22° C.

FIG. 71.—Colony of *ps. aeruginosa* after twenty-four hours on gelatin at 20°-22° C.

FIG. 72.—Colony of *ps. aeruginosa* after forty-two hours on gelatin at 20°-22° C.

Allow the chloroform extract to stand for several days; note what occurs; how do you account for it?

Prepare a 100 c.c. Ehrlenmeyer flask with 75 c.c. of sterile bouillon or peptone solution. Inoculate it with this

organism and allow it to stand, without shaking, in the incubator at body temperature for about a week. Note its condition on removal. Now agitate it thoroughly with air; best by pouring it into a beaker and stirring with a glass rod. Note what now occurs. Now abstract with 5 c.c. of chloroform—again the blue extract of “pyoscyanin” is obtained. The dirty yellowish or reddish-yellow color of the supernatant fluid, somewhat fluorescent, is due to a yellowish pigment, soluble in alcohol and water, known as “fluorescin.”

Cultivate the organism in one or another of the synthesized media—Fränkel’s modification of Uschinsky’s medium, for instance:

Water distilled	1000 c.c.
Asparagin	4 grams
Ammonium lactate	6 grams
Hydrogen Sod. phosphate (Na_2HPO_4)	2 grams
Sodium chloride	5 grams

Does it produce any color? Is chloroform extract of such cultures colored? How do you explain the result?

Obtain from the water or the soil an organism that in several particulars suggests *B. pyocyaneus*, namely, *bacillus fluorescens liquefaciens*. Repeat the foregoing cultivations and tests. In what way do the results differ from those obtained with *B. pyocyaneus*?

Make two bouillon cultures of *bacillus fluorescens liquefaciens*. Place one in the incubator and keep the other at room-temperature. How do they differ at end of 48 hours?

Its growth in gelatin-stab-cultures is accompanied by liquefaction and the diffusion of a bright-green color throughout the surrounding unliquefied medium. As

liquefaction continues, and the whole of the gelatin ultimately becomes fluid, the green color is confined to the superficial layers in contact with the air. The form taken by the liquefying portion of the gelatin in the earliest stages of development is somewhat that of an irregular slender funnel. (See Fig. 70.)

On gelatin plates the colonies develop rapidly; they are not sharply circumscribed, but usually present at first a fringe of delicate filaments about their periphery. (See Fig. 71.) As growth progresses and liquefaction becomes more advanced the central mass of the colony sinks into the liquid, while at the same time there is an extension of the colony laterally. At this stage the colony, when slightly magnified, may present various appearances, the most common being that shown in Fig. 72.

The gelatin between the growing colonies takes on a bright yellowish-green color; but as growth is comparatively rapid, it is quickly entirely liquefied, and one often sees the colonies floating about in the pale-green fluid.

On agar-agar the growth is dry, sometimes with a slight metallic lustre, and is of a pale gray or greenish-gray color, while the surrounding agar-agar is bright green. With time this bright green becomes darker, passing into blue-green, and finally turns almost black.

On potato the growth is brownish, dry, and slightly elevated above the surface. In some cultures the potato about the line of growth becomes green; in others this change is not so noticeable. With many cultures a peculiar phenomenon, consisting of a change of color from brown to green, may be produced by lightly touching the growth with a sterile platinum needle. The change occurs only at the point touched. It is best seen in cultures that have been

kept in the incubator for from seventy-two to ninety-six hours. It occurs in from one to three minutes after touching with the needle, and may last for from ten minutes to a half-hour. This is the "chameleon phenomenon" of Paul Ernst.

In bouillon the green color appears, and the growth is seen in the form of delicate flocculi. A very delicate mycoderma is also produced. As growth progresses, the bouillon becomes darker and darker in color, and more or less fluorescent, until it finally is about comparable in this respect to crude petroleum; at the same time it assumes a peculiar ropiness, and very old cultures (four to six weeks in the incubator) may attain about the consistency of raw egg-albumen. This is due to the production of a substance closely allied, chemically speaking, to mucin. Whether it is a metabolic product or one resulting from the degeneration or the autodigestion, so to speak, of the bacteria, cannot now be said; at all events, in cultures presenting this peculiarity very few bacteria of normal appearance—indeed, very few bacteria at all—are to be seen on microscopic examination.

In milk it causes an acid reaction, with coincident coagulation of the casein.

On blood-serum and egg-albumen its growth is accompanied by liquefaction. The growth on coagulated egg-albumen is seen as a dirty-gray deposit surrounded by a narrow brownish zone; the remaining portion of the medium is bright green in color. As the culture becomes older the green may give way to a brown discoloration.

In peptone solution it causes a bluish-green color. In one of four cultures from different sources we observed the production of a distinct blue color. In another specimen the fluid was of a distinct wine red color, after 5 days at body temperature.

It produces indol.

It stains with the ordinary dyes, and its flagella may readily be demonstrated by appropriate methods of staining.

It is an active producer of a proteolytic enzyme that may readily be separated and its digestive properties observed by the following simple method: Prepare a bouillon culture of about 70 to 80 c.c. volume, and allow it to grow at 37° to 38° C. for four or five days. Filter through a Berkefeld filter into a sterile receiver. Under aseptic precautions decant the filtrate into sterile test-tubes, about 7 c.c. to each tube. Then under aseptic precautions make the following tests: To one tube add a small bit of hard-boiled egg (about one-half the size of a pea) and place in an incubator. Render another tube slightly acid with dilute hydrochloric acid, and add a bit of the white of egg to it also. Do the results differ?

Heat another tube to 80° C. for fifteen minutes, and repeat the experiment. Has the heating had any effect?

To another tube add carbolic acid to the extent of 2 or 3 per cent. Is the digestive activity of the solution modified?

To two ordinary tubes of gelatin add carbolic acid until it is present to the extent of 0.25 per cent. in each tube. Solidify the gelatin in one tube in the upright position; let that in the other remain fluid. On the surface of the former pour 0.5 c.c. of the pyocyanus filtrate, and mark the point of contact between the gelatin and filtrate. To the other tube add a similar amount of filtrate, mix thoroughly, and solidify in a glass of cold water.

At the end of eighteen to twenty hours note result. Is it possible to solidify again the gelatin through which the filtrate was mixed, by placing the tube in cold water?

Do the activities of this enzyme suggest those of any of

the enzymes encountered in the animal body? Which? and Why?

Extract with chloroform a six days' old bouillon culture of this organism. In which portion of the liquid so extracted is the proteolytic ferment contained, the chloroform extract or the supernatant fluid?

Mix slowly a two weeks' old bouillon culture of this organism, grown at body temperature, with six times its volume of absolute alcohol. Allow to stand over night. Filter. Redissolve the precipitate in a few c.c. (5 or 6), of physiological salt solution. In the meantime evaporate the alcohol filtrate to dryness at a temperature not exceeding 40° C., and redissolve the sediment in 5 or 6 c.c. of physiological salt solution. Test both of these solutions on carbolized gelatin for proteolytic activity. What are the results and how are they explained?

Inoculation into Animals.—As a rule, cultures of this organism obtained directly from the discharges of the wound are capable, when introduced into animals, of producing diseased conditions; but cultures kept on artificial media for a long time may in part, or completely, lose this power.

When guinea-pigs or rabbits are inoculated subcutaneously with 1 c.c. of virulent fluid cultures of this organism, death usually results in from eighteen to thirty-six hours. At the seat of inoculation there are found an extensive purulent infiltration of the tissues and a marked zone of inflammatory edema.

When introduced directly into the peritoneal cavity the results are also fatal, and at autopsy a genuine fibrinous peritonitis is found. There is usually an accumulation of serum in both the peritoneal and pleural cavities. At autopsies after both methods of inoculation the organisms

will be found in pure cultures in the blood and internal viscera.

When animals are inoculated with small doses (less than 1 c.c. of a bouillon culture) of this organism death may not ensue, and only a local inflammatory reaction (abscess-formation) may be set up. In these cases the animals are usually protected from subsequent inoculation with doses that would otherwise prove fatal.

Most interesting in connection with *pseudomonas æruginosa* is the fact, as brought out in the experiments of Bouchard, and of Charrin and others, that its products possess the power of counteracting the pathogenic activities of *bacterium anthracis*. That is to say, if an animal be inoculated with a virulent anthrax culture, and soon after be inoculated with a culture of *pseudomonas æruginosa*, the fatal effects of the former inoculation may be prevented. Emmerich and Löw¹ are inclined to attribute this to the direct bacteriolytic action of the *enzymes* upon the anthrax bacteria introduced into the tissues.

In the literature upon the green-producing organisms that have been found in inflammatory conditions several varieties—believed to be distinct species—have been described; but when cultivated side by side their biological differences are seen to be so slight as to render it probable that they are but modifications of one and the same species.

BACILLUS PESTIS, YERSIN, 1894. THE BACILLUS OF BUBONIC PLAGUE.

Before passing from the subject of suppuration it may not be inappropriate to call attention to the light that

¹ Münchener med. Wochenschrift, 1898, No. 40; Centralblatt für Bakteriologie und Parasitenkunde, 1899, Abt. i, No. 1, p. 33.

modern methods of investigation have shed upon the etiology of bubonic plague, an epidemic disease characterized by suppuration of the lymphatic glands, and accompanied by a very high rate of mortality, especially when the infection involves the lungs, as is sometimes the case.

This pestilence, probably endemic in certain sections of the Orient, is one of the most conspicuous epidemic diseases of history. Since early in the Christian era epidemics and pandemics of plague have made their appearance in Europe at different times. During and for a time after the Middle Ages it was more or less frequent in India, China, Arabia, Northern Africa, Italy, France, Germany, and Great Britain. In history it is variously known as the "Justinian Plague" of the sixth century, the "Black Death" of the fourteenth century, and the "Great Plague of London" of the seventeenth century, though it is difficult to say to what extent these outbreaks were uncomplicated manifestations of genuine bubonic plague. During the existence of the Justinian Plague 10,000 people are said to have died in Constantinople in a single day, and Hecker estimates that during the pandemic of the Black Death 25,000,000 people (a quarter of the entire population of Europe) succumbed to the disease. During the Great Plague of London (1664-65) the total mortality for one year was 68,596, out of an estimated population of 460,000 souls.

It is not surprising to learn that it was to guard against the plague that quarantine regulations were first established.

The first and certainly the most exact information concerning the exciting cause and the pathology of the plague was furnished by investigations of Yersin, of Kitasato, and of Aoyama, conducted during the epidemic of 1894 in Hong

Kong, China; although since then numerous other investigators have made additional important contributions to our knowledge of the subject. The results of these studies demonstrate that bubonic plague is an infectious, not markedly contagious disease (except in the case of the pulmonic variety), that depends for its existence upon the presence in the tissues of a specific micro-organism—the so-called plague or pest bacillus.

This organism is described as a short, oval bacillus, usually seen single, sometimes joined end to end in pairs or threes, less commonly as longer threads. It stains more readily at its ends than at its centre. It is sometimes capsulated; is non-spore-forming; is aërobic, and is non-motile. It is found in large numbers in suppurating glands. (Fig. 73.) It is also to be detected in the blood, spleen, lungs, liver, kidneys, walls of the stomach and intestines, urine, and intestinal contents of fresh cadavers; and during life in the blood, expectorations, feces, and urine of persons sick of the disease. From these findings the infection is obviously a septicemia.

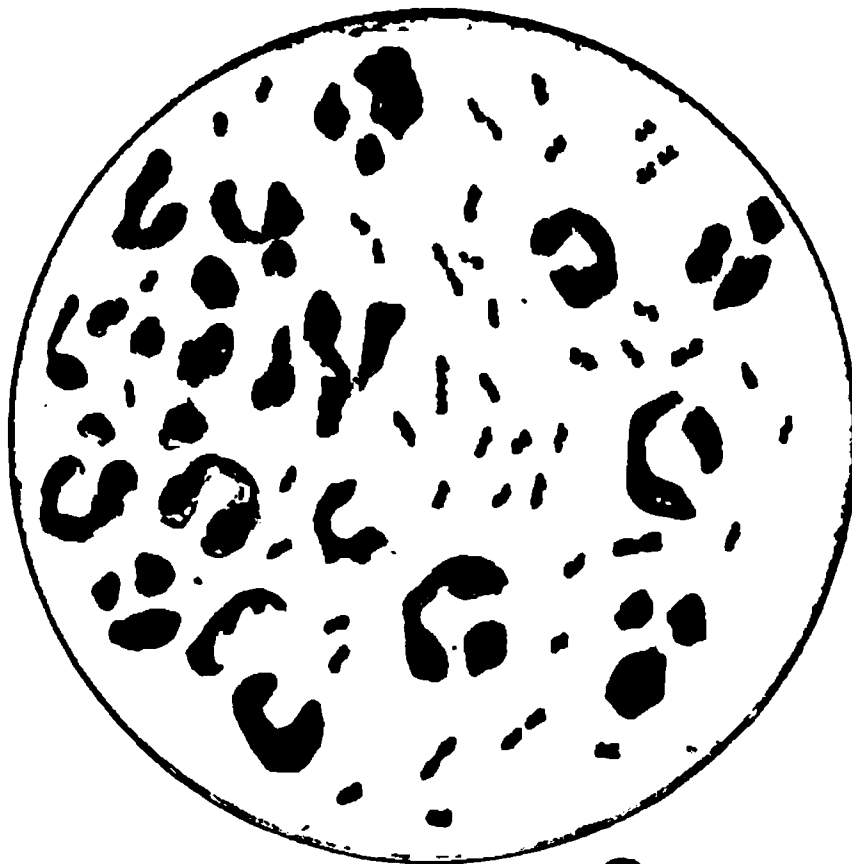
It is negative to the Gram method but stains readily with the ordinary aniline dyes. It may be cultivated upon ordinary nutrient media, although preference is given by some to a neutral or slightly alkaline 2 per cent. peptone solution containing from 1 to 2 per cent. of gelatin.

The most favorable temperature for its growth is between 36° and 39° C. Its colonies on glycerin-agar-agar and on coagulated blood-serum are described as iridescent, transparent, and whitish. On gelatin at 18°–20° C. it develops as small, sharply defined, white colonies without liquefaction of the medium. In stab-cultures it develops both on the surface and along the track of the needle. Its growth is

slow. It does not cause a diffuse clouding of bouillon, but grows rather as irregular, flocculent clumps that adhere to the sides or sink to the bottom of the vessel, leaving the fluid clear. It shows but limited growth on potato. It does not ferment glucose with production of gas, nor does it form indol. It coagulates milk.

FIG. 73

A



B

Bacillus of bubonic plague: A, in pus from suppurating bubo; B, the bacillus very much enlarged to show peculiar polar staining.

This organism is killed by drying at ordinary room-temperature in four days. It is killed in three or four hours by direct sunlight. It is destroyed in a half hour by 80° C., and in a few minutes by 100° C. (steam). It is killed in

one hour by 1 per cent. carbolic acid and in two hours by 1 per cent. milk of lime.¹

It is pathogenic for rats, mice, guinea-pigs, ground squirrels, rabbits, hogs, horses, monkeys, cats, chickens, and sparrows. Pigeons, hedgehogs, and frogs are immune, and dogs and bovines are apparently so.²

Animals succumb to subcutaneous inoculation in from two to three days. According to Yersin, the site of subcutaneous inoculation becomes edematous and the neighboring lymphatics are enlarged in a few hours. After twenty-four hours the animal is quiet, the hair is ruffled, tears stream from the eyes, and later convulsions set in, which last till death. The results found at autopsy are: blood-stained edema at the site of inoculation, reddening and swelling of the lymphatic glands, bloody extravasation into the abdominal walls, serous effusion into the pleural and peritoneal cavities; the intestine is occasionally hyperemic, the adrenal bodies congested, and the spleen enlarged, often being studded with grayish points, suggestive of miliary tubercles. The plague, or pest, bacillus is detected in large numbers in the local edema, the lymph-glands, the blood, and the internal organs.

As is the case in general with the group of hemorrhagic septicemia bacteria, the members of which it resembles in certain other respects, when death does not result promptly after infection there is usually only local evidence of the inoculation, the distribution of the micro-organisms throughout the body being considerably diminished.

Animals that survive inoculation with this organism

¹ See Viability of the *Bacillus Pestis*, by M. J. Rosenau, U. S. Marine-Hospital Service, Bulletin No. 4, of the Hygienic Laboratory, U. S. M.-H., Washington, D. C., 1901.

² Nuttall, *Centralblatt für Bakteriologie und Parasitenkunde*, 1897, Abt. 1, Bd. xxii, S. 97.

usually exhibit a certain degree of immunity from subsequent infection.

Nuttall¹ notes that feeding-experiments have resulted in fatal infection in gray and white rats, house- and field-mice, guinea-pigs, rabbits, hogs, apes, cats, chickens, sparrows, and flies. He also calls attention to the fact that flies may live for several days after being infected with this organism, and if at liberty to fly about may infect persons or foodstuffs on which they alight or fall.

Investigations in India demonstrate that the flea is the most common and important of the agents of transmission, carrying the disease from man to animals (rodents, rats in particular) and from animals to man.

The bacilli apparently lose their virulence after long-continued cultivation under artificial conditions, and it is said that from slowly developing, chronic buboes non-virulent or feebly virulent cultures are often obtained. Variations in the degree of virulence have been observed in different colonies from the same source. Virulence is said by Yersin, Calmette, and Borrel² to be accentuated by passing the organism through a series of susceptible animals.

It has been observed that in the suppurating lymphatic glands of man a variety of organisms may be present, but among them are always the plague bacilli. Occasionally micrococci predominate. In these cases of mixed infection the pest bacilli are said to stain less intensely with alkaline methylene-blue than do the streptococci, and more intensely than do the micrococci that are present. Also, in this event, the streptococci retain the Gram stain, while the pest bacilli do not and the staphylococci may or may not. It has been

¹ Loc cit.

² Annales de l'Institut Pasteur, 1895, p. 589.

suggested that possibly the organisms found by Kitasato in the blood, and which he describes as pest bacilli, that retained the color when treated by the method of Gram, were pairs of micrococci, and not bacilli at all.

It is the opinion of Aoyama that the suppuration of the glands is not caused by the plague bacillus, but is rather the result of the action of the pyogenic cocci with which it is so often associated. It is also his belief that the most important and frequent mode of infection in man is through wounds of the skin. He does not regard either the air-passages or the alimentary tract as frequent portals of infection. Wilm, on the contrary, is inclined to regard the alimentary tract as a frequent portal of infection;¹ and there is a general concensus of opinion that in the pulmonic type of plague, its most fatal manifestation, infection is always by way of the respiratory tract.

The order in which the lymphatics manifest disease appears to depend upon the location of the primary infection. That is to say, if it is upon the feet, as of persons who go barefooted, the superficial and deep inguinal glands are the first to show signs of the disease; while if infection occurs through wounds of the hand, the buboes appear first in the axillary region. As a rule, the wound through which infection is received shows little or no inflammatory reaction.²

Wyssokowitz and Zabolotny³ call attention to the fact that the blood of patients convalescing from plague has an agglutinating action upon fluid cultures of the plague bacillus analogous to that observed when the blood-serum of typhoid

¹ Wilm, *Hyg. Rundschau*, 1897, p. 217.

² The works of Yersin, of Kitasato, and of Aoyama have been exhaustively reviewed by Flexner in the *Bulletin of the Johns Hopkins Hospital*, 1894, v, 96, and 1896, vii, 180. I am indebted to these reviews for much that is here presented on this subject.

³ *Annales de l'Institut Pasteur*, 1897, p. 663.

or of cholera patients is mixed with similar cultures of the typhoid or the cholera bacillus. (See Agglutinins).

Protective Inoculation; Vaccination.—Active immunization from plague infection by protective inoculation has been variously attempted; by subcutaneous or intramuscular injection of old bouillon cultures of bacillus pestis that had been killed by heat; by similar injections of emulsions made from agar-agar cultures of different ages suspended in isotonic salt solution and likewise killed by heat; by the injection of determined amounts of extractives from plague bacilli; by the injection of mixtures of dead plague bacilli and plague immune serum; by injection of the filtrate from fluid cultures of the organism; by the injections of peritoneal exudates and organ extracts of animals infected with plague; and by the injection of attenuated living cultures of the organism. For the most part these efforts have been experimental, that is to say, they have been made upon animals susceptible to plague infection, notably guinea-pigs and monkeys. In the problem of protecting human beings from plague, dead cultures have been used practically to the exclusion of all other methods. The method of Haffkine¹ has enjoyed more favor than any of the others, though it is difficult to determine its protective value with any degree of exactness.² This method consists in the subcutaneous injection of from 0.5 c.c. to 7.0 c.c. of a six weeks' old, specially prepared bouillon culture of bacillus pestis that had been killed by exposure to 65° C. for one hour. Sometimes the smaller, sometimes the larger doses are indicated; sometimes a single injection is given, sometimes several are repeated at shorter or longer intervals according to circum-

¹ British Med. Jour., 1897, No. 12.

² Bull. de l'Institut Pasteur, 1906, No. 4, p. 825.

stances. The injections are followed by both local and constitutional reactions, varying in the degree of intensity and length of duration with different individuals. The immunity resulting is said to be established fairly promptly and to last for six weeks and longer. The investigations of the Indian Plague Commission justify the conclusion that both morbidity and mortality for Plague is less among the inoculated than among the uninoculated.

In so far as experiments upon animals and observations upon human beings afford positive light on this subject, the protective inoculations protect only against the bubonic type of plague and are practically without influence in preventing the pulmonary or pneumonic manifestation.

The comprehensive critical review of this subject made by Strong¹ led him to the same conclusion as that of Kolle and Otto:² that the most effective protection from plague is that afforded by the injection of attenuated, living cultures. Tests made upon monkeys and guinea-pigs demonstrated this method to be, in round numbers, three times as effective as when cultures killed by heat are used. While the results of these investigations fully warrant the conclusions drawn by the authors, it is doubtful if the method will be generally approved as applicable to man. The possibility of accident where living cultures are used even though they be attenuated to the point of harmlessness, as decided by animal tests, is more than likely to operate against the routine employment of such cultures in the protection of human beings by vaccination.

Besredka, of the Pasteur Institute,³ advocates the use

¹ Philippine Journal of Science, Section B, 1907, p. 155; 1912, p. 223.

² Zeit. f. Hyg. Infektionskr., 1903, S. 45.

³ Bull. de l'Institut Pasteur, 1910, viii, p. 241; 1912, x, p. 529.

of a "sensitized vaccine" against plague. This consists of dead pest bacilli (killed by heat) that have been mixed with antiplague immune serum obtained from an artificially immunized animal. It is claimed by him that the process of sensitizing lessens the toxic action of the dead bacteria; diminishes the risk run by injecting them and eliminates the uncomfortable local and constitutional reactions that so often accompany the injections; while at the same time the protective properties of the "vaccine" are preserved. Rowland,¹ in a critical review of the subject, fails to find any neutralization of the toxic properties of the dead bacteria through sensitization, but states that Besredka's "vaccine" possesses good immunizing power and users of it have reported favorably as to the minimum of discomfort following its inoculation. The principle here used has been applied by Besredka, Gay and others to the making of protective agents for other types of infection.

Antiplague Serum.—The general principles that are involved in the induction of immunity with antibody formation hold for plague as for a number of other types of infection; that is to say, the repeated injection into animals of non-fatal doses of the specific organism or the products of its growth, results in the elaboration in the injected animal of substances that are in one way or another antidotal, destructive or neutralizing for the matters injected.

In the effort to secure specific antiplague serum two general plans have been followed: one, by the repeated injection of horses with at first increasing doses of dead pest bacilli followed by ascending doses of the living organism (Yersin's method);² the other by the injection of the

¹ Journal of Hygiene, Plague Supplement II, 1912, p. 344.

² Annales de l'Institut Pasteur, 1897, p. 81.

toxic extractives from artificially treated plague bacilli (the method of Lustig¹). The former method aims to establish an antibacterial immunity, the latter an antitoxic immunity.

By both modes of procedure sera are obtained that possess some degree of curative value in the treatment of plague, but in both instances this is low. When tested on human beings sick of plague under as well controlled conditions as are offered by a good hospital, it was concluded by the Indian Plague Commission:² "From the whole inquiry therefore it appears that the administration of the available sera is not a practicable means of bringing about any material diminution in the mortality from plague in India. It may well be that better results would be obtained if the treatment could be commenced within a few hours of the onset of the disease, this however, is in the great majority of cases, impossible in ordinary practice."

The investigations of the Pest Commissions of Germany, Anstria, and Egypt, as well as those of the Institutes for Infectious Diseases at Berlin, Berne, and the Pasteur Institute of Paris,³ have contributed much additional information of importance to this subject. They confirm the original views upon the protective or prophylactic value of the antiplague serum, but demonstrate that as a therapeutic agent it is of but limited usefulness.

¹ *Deutsche med. Woch.*, 1897, No. 15.

² *Journal of Hyg., Plague Supplement II, Seventh Report*, 1912, p. 326.

³ The important literature bearing on this subject is appended to the report of Kolle, Hetsch, and Otto (*Zeitschr. f. Hygiene*, Bd. xlviii, p. 368).

CHAPTER XX.

Some of the Pathogenic Organisms Encountered in the Mouth Cavity in Health and Disease—*Micrococcus Lanceolatus*, *Micrococcus Tetrigenous*, *Bacterium Influenzæ*, *Bacillus Tuberculosis*, etc.

USUALLY in the course of certain diseases, and from time to time in health, pathogenic bacteria are to be found in the mouth. In the latter instance the organisms, while often fully pathogenic, as shown by tests on animals, do apparently no harm to their hosts, with whom they live in a commensal relationship. Moreover, they are often not regularly and persistently present—at times they may disappear permanently, at other times they may be recurrent, with varying intervals, for longer and shorter periods. The typical “pneumococcus,” as it is called; the micrococcus tetragenous; the influenza bacillus, the bacillus diphtheriæ, and the ordinary pyogenic streptococci may be cited as occasional guests in the normal mouth cavity. In diphtheria, tonsillitis, influenza and tuberculosis, the specific organisms of these diseases may usually be detected either in the ordinary saliva or in the sputum brought up from the deeper respiratory tract.

To familiarize one's self with these organisms and the customary technique for their isolation one may proceed as follows:

Obtain from a tuberculous patient a sample of fresh sputum—that of the morning is preferable. Spread it in a thin layer upon a black glass plate and select one of the small, white, cheesy masses or dense mucous clumps scat-

tered through it. With a pointed forceps smear this carefully upon two or three thin cover-slips, dry and fix them in the way given for ordinary cover-slip preparations. Stain one with Löffler's alkaline methylene-blue solution, another by the Gram method, and a third after the method given for bacterium tuberculosis in fluids or sputum.

In that stained with Löffler's blue—slip No. 1—will be seen a great variety of organisms—round cells, ovals, short and long rods, perhaps spiral forms. But not infrequently will be seen diplococci having more or less of a lancet shape, joined together by their broad ends, the points of the lancet being away from the point of juncture of the two cells. There may also be seen masses of cocci which are conspicuous by their arrangement into groups of fours, the adjacent surfaces being somewhat flattened.

In the slip stained by the Gram method the same groups of cocci which grow as threes and fours will be seen; but the lancet-shaped diplococci may now present an altered appearance—they are usually surrounded by a capsule. This capsule is very delicate in structure, and, though a frequent accompaniment, is not constant. It can sometimes be demonstrated by the ordinary methods of staining, though the method of Gram is most satisfactory. (Fig. 75.)

In the third slip, which has been stained by the method given for tubercle bacteria in sputum, if decolorization has been properly conducted and no contrast-stain has been employed, the field will be colorless or of only a very pale rose color. None of the numerous organisms seen in the first slip can now be detected; but instead there will be seen scattered through the field very delicate, stained rods, which present, in most instances, a conspicuous beading of their protoplasm—that is, the staining is not homogeneous,

but at tolerably regular intervals along each rod are seen alternating stained and unstained points. These rods may be found singly, in groups of twos and threes, and sometimes in clumps consisting of large numbers. When in twos or threes it is not uncommon to find them describing an X or a V in their mode of arrangement, or again they may be seen lying parallel the one to the other.

If contrast-stains are used, these rods will be detected and recognized by their retaining the original color with

FIG. 74



Tuberculous sputum stained by Gabbett's method. Tubercle bacteria seen as red rods; all else is stained blue.

which they had been stained; whereas all other bacteria in the preparation, as well as the tissue-cells which are in the sputum, will take up the contrast-color. (Fig. 74.)

This delicate, beaded rod is *bacterium tuberculosis*. The lancet-shaped diplococcus with the capsule is *bacterium pneumoniae*. The cocci grouped in fours are *sarcina tetragena*.

Inoculation Experiment.—Inoculate into the subcutaneous tissues of a guinea-pig one of the small, white, caseous masses, similar to that which has been examined micro-

scopically. If death ensue, it will, in all probability, be the result of one of the three following types of infection:

a. Septicemia resulting from the introduction into the tissues of *bacterium pneumoniae*.

b. A less active form of septicemia resulting from the introduction of *sarcina tetragena*, an organism frequently seen in the sputum.

c. Local or general tuberculosis.

SPUTUM SEPTICEMIA. BACTERIUM PNEUMONIAE (WEICHSELBAUM), MIGULA, 1900.

SYNONYMS: *Diplococcus pneumoniae*, Weichselbaum, 1886; *Pneumococcus*, Fränkel, 1886; *Micrococcus* of sputum septicemia; *Diplococcus lanceolatus*; *Streptococcus lanceolatus*; *Streptococcus pasteurii*; *Micrococcus lanceolatus*.

If at the end of twenty-four to thirty-six hours the animal be found dead, we may reasonably predict that the result was produced by the introduction into the tissues of the organism of sputum septicemia above mentioned, viz., *bacterium pneumoniae*, which is not uncommonly found in the mouths of healthy individuals as well as in other conditions.

Inspection of the site of inoculation usually reveals a local reaction. "This may be of a serous, fibrinous, hemorrhagic, necrotic, or purulent character. Frequently we may find combinations of these conditions, such as fibrino-purulent, fibrino-serous, or sero-hemorrhagic."¹ The most conspicuous naked-eye change undergone by the internal organs will be enlargement of the spleen. It is usually swollen, but may at times be normal in appearance. It is sometimes

¹ Welch, Johns Hopkins Hospital Bulletin, December, 1892, vol. iii, No. 27.

hard, dark red, and dry; or it may be soft and rich in blood. Frequently there is a limited fibrinous exudation over portions of the peritoneum.

Except in the exudations, the organisms are found only in the lumen of the bloodvessels, where they are usually present in enormous numbers. In the blood they are practically always free, being but rarely found within the bodies of leukocytes.

FIG. 75

Bacterium pneumoniae in blood of rabbit. Stained by method of Gram. Decolorization not complete.

In stained preparations from the blood and exudates a capsule is not infrequently seen surrounding the organisms. (Fig. 75.) This, however, is not constant.

If a drop of blood from the dead animal be introduced into the tissues of a second animal (mouse or rabbit), identically the same conditions will be reproduced.

If the organism be isolated in pure culture from the blood of the animal, and a portion of this culture be introduced into the tissues of a susceptible animal, we shall see again the same pathological picture.

It must be remembered, however, that this organism when cultivated for a time on artificial media may lose rapidly its pathogenic properties. If, therefore, failure to reproduce the disease after inoculation with old cultures should occur, it is in all probability due to such loss of virulence.

This organism was discovered by Sternberg in 1880. It was subsequently described by A. Fränkel as the etiological factor in the production of acute fibrinous pneumonia.

It is not uncommonly present in the saliva of healthy individuals, having been found by Sternberg in the oral cavities of about 20 per cent. of healthy persons examined by him, and certain authors are of the opinion that it occurs in the oral or nasal cavities of *all* individuals at one time or another during life. It is constantly to be detected in the rusty sputum of patients suffering from acute fibrinous pneumonia. Its presence has been noted in the middle ear, in the pericardial sac, in the pleura, and in the serous cavities of the brain; and indeed it may penetrate from its usual site of development in the mouth to any of the more distant organs.

The organism is commonly found as a diplococcus, though here and there short chains of four to six individuals may be seen. (Fig. 75.) The individual cells are more or less oval, or, more strictly speaking, lancet-shaped, for at one end they are commonly pointed. When joined in pairs the junction is always at the broad ends of the ovals. When in chains only the terminal cells are pointed, and then at their distal extremities.

As already stated, in preparations directly from the sputum or from the blood of animals a delicate capsule may frequently be seen surrounding them. Though fairly constant in preparations directly from the blood of animals and from

the sputum or lungs of pneumonic patients, the capsule is but rarely observed in artificial cultures. Occasionally in cultures on blood-serum, in milk, and on agar-agar it can, according to some authors, be detected; but this is by no means constant, or even frequent.

Under the most favorable artificial conditions this organism grows but slowly, and frequently not at all.

When successfully grown upon the different media it presents somewhat the following appearances:

On gelatin its development is very limited and often no growth at all occurs. This is probably due in part to the low temperature at which gelatin cultures must be kept. If development occurs, the growth appears as minute whitish or blue-white points on the plates. These very small colonies are round, finely granular, sharply circumscribed, and slightly elevated above the surface. They do not cause liquefaction of the gelatin.

If grown in slant- or stab-cultures, the surface-development is very limited; along the needle-track tiny whitish or bluish-white granules appear.

On nutrient agar-agar the colonies are almost transparent, more or less glistening, and very delicate in structure.

On blood-serum development is more marked, though still extremely feeble, appearing as a cluster of isolated fine points growing closely side by side.

Growth on potato is not usually observed.

When grown in milk it commonly causes an acid reaction with coincident coagulation of the casein. Some varieties, especially non-virulent ones, do not coagulate milk.¹

It is not motile.

¹ Welch, loc. cit.

It grows best at a temperature of from 35° to 38° C. Below 24° C. there is usually no development, but in a few cases it has been seen to grow at as low a temperature as 18° C. Above 42° C. development is checked.

It grows as well without as with oxygen. It is therefore one of the facultative anaërobic forms.

Cultivation of this organism is most successful when some one of the serum-agar or agar-gelatin mixtures is employed. (See the medium.)

It may be stained with the ordinary aniline staining-reagents. For demonstrating the capsule the method of Gram and the acetic-acid method give the best results. (See Stainings.)

This organism is conspicuous for the irregularity of its behavior when grown under artificial conditions: usually it loses its pathogenic properties after a few generations; but again this peculiarity may be retained for a much longer time. Often it fails to grow after three or four transplantations on artificial media, though at times it may be carried through many generations.

Inoculation into Animals.—The results of inoculations with pure cultures of this organism are also conspicuous for their irregularity. When the organism is of full virulence the form of septicemia above described is usually produced, but at times it is found to be totally devoid of pathogenic powers: between these extremes cultures may be obtained possessing every variation in the intensity of their disease-producing properties. The principal pathological conditions that may be produced by the inoculation of susceptible animals with this organism are, according to the degree of its virulence, acute septicemia, spreading inflammatory exudations, and circumscribed abscesses. All three of these conditions

may sometimes be produced by inoculating rabbits with the same cultures in varying amounts.

Rabbits, mice, guinea-pigs, dogs, rats, cats, and sheep are susceptible to infection by this organism. Chickens and pigeons are insusceptible. Young animals, as a rule, are more easily infected than old ones. Rabbits and mice are the most susceptible of the animals used for experimental purposes, and in testing the virulence of a culture it is well to inoculate one of each, for the same culture may sometimes be virulent for mice and not for rabbits, or *vice versa*.

If the culture is virulent, intravascular or intraperitoneal injections into rabbits may produce rapid and fatal septicemia; while subcutaneous inoculation of the same material may result in only a localized inflammatory process. On the other hand, subcutaneous inoculation of less virulent cultures may produce a local process, while intravenous inoculation may be without result.

This organism is the cause of a number of pathological conditions in human beings that are not usually considered as related to one another etiologically. It is always present in the inflamed area of the lung in acute fibrinous or lobar pneumonia; it is known to cause acute cerebrospinal meningitis, endo- and pericarditis, certain forms of pleuritis, arthritis and peri-arthritis, and otitis media.

The Mechanism of Pneumonic Infection—The most important result of pneumococcus infection in man is pneumonia. The mechanism of the origin, course and recovery from pneumonia still constitutes one of the obscure problems of medicine, even though special investigations have shed much light upon several important phases of the subject.

For a clear appreciation of the current views on the

essential features of this riddle, we must bear in mind several fundamental facts:

1. That pneumonia is not invariably the consequence of the presence of pneumococci upon the mucous surfaces or in the body, for that organism is often found, fully virulent in the mouth, nose or upper air passages of persons in perfect health.

2. That pneumonia, when not terminating fatally, is a self-limited disease, *i. e.*, the signs and symptoms increase from the start until a point is reached, "the crisis," when their severity suddenly begins to lessen and may continue to do so until recovery is established.

3. That up to, and for a time after the crisis, often far into convalescence, living virulent pneumococci are present in the lungs. They can be found constantly in the sputum and often in smaller or larger numbers in the circulating blood. Their number seems at times to be affected little, if at all, by the forces that occasion the crisis.

4. That the pathogenic activities of the pneumococcus are not referable to an extracellular toxin, properly so called, but rather to an endotoxic component that is liberated in the body when the bacteria are disintegrated and that may be liberated artificially by certain solvents and under such conditions as favor autolysis, *i. e.*, by the self-digestion of the bacteria.

5. That in the blood of convalescents from pneumonia specific, protective antibodies are to be found, but as they are inconstant both as to their presence and as to their amounts it is impossible to decide their rôle in the mechanism of recovery.

6. That animals may be actively immunized from pneumococcus infection with but little difficulty, but the serum

from such animals is not always of value in either preventing infection in other animals in which it is injected or of mitigating or curing infections already established in animals.

It is only by keeping in mind the foregoing facts that we are able to appreciate the difficulties surrounding the problem of pneumonia or to properly estimate the value of certain important experimental results having a bearing upon it.

Given a group of persons with virulent pneumococci in their mouths, noses and pharynges, why is it that some may develop pneumonia and others remain in health?

It has been customary to reply: that in those developing the disease there has been a lessening of the general vitality (resistance) through a variety of agencies, to a point that enables the pneumococcus, hitherto present only in a commensal relationship, to exhibit its pathogenic activities. This is plausible, but that is all. There is nothing definite in the way of experimental evidence to support it.

The most satisfying explanation of the beginnings of pneumonia is that offered by the investigations of Meltzer¹ and his associates. They demonstrated that if fairly large amounts (5 or 6 c.c.) of fluid cultures of pneumococci be insufflated into the lungs of dogs, that many of the bronchioles became occluded as the result of the exudation following such insufflations. The occlusion converts the termini of those bronchioles, with their alveoli, into tiny cavities. In such cavities the pneumococci develop and produce irritating substances which in time bring about more or less extensive inflammation of the lung tissues round about them. The characteristics of these inflamma-

¹ Jour. Exp. Med., 1912, xv, 133.

tory areas are in all important details identical with those of true pneumonia in man. This experimentally-produced pneumonia is not, however, clinically identical with pneumonia in man, as it is not accompanied by the crisis, nor does one observe the sequence of local changes leading to resolution that are commonly noticed in the course of pneumonia in man. Nevertheless, the results of this investigation justify the conception that pneumonia in man may not, after all, be from the start a matter purely and simply of the invasion of the lung by pneumococci, but rather that for such invasion to be followed by the characteristic lesions of the disease, there must first exist physical conditions favorable to the massed or circumscribed development of the organism. In the light of Meltzer's studies one can conceive that through one or another of many causes exudations, non-specific in character, may occur in the lungs, occlude terminal bronchi and, as in the experimental cases, cause small cavities into which pneumococci, gaining access, develop as in a closed space—and by the products of their growth bring about progressive inflammation of the tissues surrounding them. The experimental evidence also suggests the view that pneumonia probably always starts as such isolated patches which, by extension, coalesce until finally larger areas or indeed whole lobes of the lungs are involved. When this inflammation of the lung, with its accompanying symptoms, have progressed for about a week, the crisis may be expected, *i. e.*, the distressing symptoms become more or less suddenly relieved, fever begins to decline, respiration is less difficult, and there are beginning signs of changes in the diseased lung tissue, *i. e.*, resolution may set in.

These sudden changes for the better, so often observed

in true lobar pneumonia, and as said, denominated "the crisis," constitute one of the dramatic phenomena of clinical medicine. As if by magic, often within a few hours, a patient apparently *in extremis*, may be found in comparative comfort and progressing steadily to recovery with little or no return of the distressing symptoms. It is needless to say that this is not the history of every case, but it is so frequently seen in non-fatal cases as to fairly characterize the course of a case destined to recover.

What are the forces that work this remarkable change for the better? It cannot be that the pneumococci causing the trouble are suddenly killed off and their hurtful action in this way terminated; for we have seen that long after the crisis they may be found in the sputum of the patient alive, fully virulent and in almost countless numbers. It has been suggested that after about a week there develops in the tissues of the body a sufficient amount of antibodies to neutralize the poison of the pneumococci and that coincident with this neutralization there is a cessation of the evil effects, *i. e.*, the crisis occurs. Vague as this may appear it is probably as satisfactory as any other explanation available at this time. There are objections or criticisms that may, however, be offered in discussing it. If that be the correct explanation of the crisis, one might reasonably expect to detect in the blood of convalescents from pneumonia protective antibodies in sufficient amount and with such constancy as to support the view, but such is not always the case. In some instances antibodies are found in the blood immediately after the crisis in such amounts that a fraction of 1 cubic centimeter of the serum will protect a mouse from infection by a hundred fold the ordinary fatal dose of virulent pneumococci; in other cases no such

protective bodies are to be demonstrated at all; in the majority of cases a certain amount of such protective agents is to be demonstrated. In some cases protective bodies may be detected in the blood a few hours after the crisis, and none may be found a few days later. It is such inconstancies as these that call into question the explanation offered above, or at least justify the suspicion that the crisis may be dependent upon other factors in addition to those having to do with the neutralization of poison or the destruction of a certain number of the germs.

It has been suggested that such other factors may comprise provisions for preventing further growth of the pneumococci in the tissues without actually killing them or robbing them of their power to produce infection when removed alive from the pneumonic patient.

It also has been suggested that the crisis constitutes the advent of a refractory state on the part of the tissues—a state having some analogies to anaphylactic shock. As yet this can be taken only as a suggestion. Much more in the way of experimental evidence is needed before it can be accepted.

It is scarcely suitable to a book of this character to pursue all the lines of argument that have been advanced in connection with this subject. It suffices to say that at present we are forced still to speculate as to the nature of at least some of the important factors responsible for the self limitation of this disease.

Immunization and Specific Antisera.—Little difficulty has been experienced in the efforts to actively immunize animals from pneumococcus infection. Horses have been carried to such a high degree of immunization by repeated intravenous injection of pneumococcus cultures that as much as

2500 c.c. of a virulent culture has been injected into the veins at one time.

From such highly immunized animals sera have been obtained of remarkable potency in preventing infection; thus Cole found that 0.2 c.c. of serum from one of his immunized horses would protect a mouse from a million-fold the lethal dose of virulent pneumococci, provided the serum and the culture be injected into the animal at the same time. But if the animal be first infected, then the serum has practically no saving powers even though it be injected only a few hours later and in very much larger amounts; in fact, Cole states, it is difficult or impossible to rescue the animal, no matter how much serum is injected.

We see then that while active immunization is comparatively easy of accomplishment, the matter is altogether different when the serum of animals so immunized is used for therapeutic purposes. The failure of serum from immunized animals to assist in the cure of pneumonia or other pneumococcus infection with certainty is variously explained, but as yet none of the explanations are universally accepted. By some it is believed that immune serum has not been used in sufficient quantities; by others it is believed that if the intensity of the infection exceeds a certain degree that no amount of immune serum will suffice to rescue. This latter view is particularly applicable to pneumonia, a disease in which one is dealing with an unusually severe type of infection associated with enormous numbers of bacteria in the body.

Cole suggests that the failure of immune serum to exhibit its curative powers in the cure of pneumonia may not be due to too small amounts of serum used, but rather to an inability on the part of the infected body to supply the

factors necessary to complement the action of the serum. His studies furthermore indicate that an essential for a curative serum for pneumonia may, after all, be analogous to that of curative sera for streptococcus infections—that is to say, the animal supplying the serum should have been immunized with a strain of pneumococcus closely related to that with which the patient to be treated is infected. His investigations lead him to several important conclusions, among which may be mentioned: Since pneumococci may be divided into several distinct groups, it is necessary to use for curative purposes a serum from an animal immunized from a strain of pneumococci belonging to the same group as that with which the patient is infected. In order to be effective antipneumococcus serum must be administered early and in large doses. With these facts in mind the treatment of human beings suffering from pneumonia with homologous, immune serum has resulted in very low mortality. In cases so treated the bacteria in the blood are destroyed and specific immune substances appear in the blood very promptly after the injection of the serum. A part of the action of the immune serum seems to be anti-toxic.¹

INFECTION WITH *SARCINA TETRAGENA* (GAFFKY), MIGULA, 1900.

SYNONYM: *Micrococcus tetragenus*, Gaffky, 1883.

Should the death of the animal not occur within the first twenty-eight to thirty hours after inoculation, but be postponed until between the fourth and eighth day, it may

¹ Cole, Jour. Am. Med. Assoc., 1912, lix, 693 and 1913, xli, 663.

result from the invasion of the tissues by the organism now to be described, viz., *sarcina tetragena*.

This organism was discovered by Gaffky, and was subsequently described by Koch in the account of his experiments upon tuberculosis. It is often present in the saliva of healthy individuals and is commonly present in the sputum of tuberculous patients. Koch found it very frequently in the pulmonary cavities of phthisical patients. It, however, plays no part in the etiology of tuberculosis. It is principally of historic interest, being of little pathogenic significance.

It is a small round coccus of about 1μ transverse diameter. It is seen as single cells, joined in pairs, and in threes; but its most conspicuous grouping is in fours, from which arrangement it takes its name. In preparations made from cultures of this organism it is not rare to find single bodies which are much larger than the other individuals in the field. Close inspection reveals them to be cells in the initial stage of division into twos and fours. A peculiarity of this organism is that the cells are bound together by a transparent gelatinous mass.

When cultivated artificially it grows very slowly.

Upon gelatin plates the colonies appear as round, sharply circumscribed, punctiform masses which are slightly elevated above the surface of the surrounding medium. Under a low magnifying power they are seen to be slightly granular and to present a more or less glassy lustre.

The colonies increase but little in size after the third or fourth day. If cultivated as stab-cultures in gelatin, there appears upon the surface at the point of inoculation a circumscribed white point, slightly elevated above the surface and limited to the immediate neighborhood of the point of inoculation. Down the needle-track the growth is not

continuous, but appears in isolated, round, dense white clumps or beads, which do not develop beyond very small points.

It does not liquefy gelatin.

Upon plates of nutrient agar-agar the colonies appear as small, almost transparent, round points, which have about the same color and appearance as a drop of egg-albumen; they are very slightly opaque. They are moist and glistening. They rarely develop to an extent exceeding 1 to 2 mm. in diameter.

Upon agar-agar as stab- or slant-cultures the surface growth has more or less of a mucoid appearance. It is moist, glistening, and irregularly outlined. The outline of the growth depends upon the moisture of the agar-agar. It is slightly elevated above the surface of the medium.

In contradistinction to the gelatin stab-cultures, the growth in agar-agar is continuous along the track of the needle.

The growth on potato is a thick, irregular, slimy-looking patch.

The transparent mucilaginous substance which is seen to surround these organisms renders them coherent, so that efforts to take up a portion of a colony from the agar-agar or potato cultures result usually in drawing out fine, silky threads, consisting of organisms imbedded in the mucoid material.

The organism grows best at from 35° to 38° C., but can be cultivated at the ordinary room-temperature—about 20° C.

The growth under all conditions is slow.

It grows both in the presence of and without oxygen.

It is not motile.

It stains readily with all the ordinary aniline dyes.

In tissues its presence is readily demonstrated by the staining-method of Gram.

The grouping into fours is particularly well seen in sections from the organs of animals dead of this form of septicemia. In such sections the organisms will always be found within the capillaries.

INOCULATION INTO ANIMALS.—To the naked eye no alteration can be seen in the organs of animals that have died as a result of inoculation with *sarcina tetragena*; but microscopic examination of cover-slip preparations from the blood and viscera reveals the presence of the organisms throughout the body—especially is this true of preparations from the spleen. White mice and guinea-pigs are susceptible to the disease. Gray mice, dogs, and rabbits are not susceptible to this form of septicemia. Subsequent inoculation of healthy animals with a drop of blood, a bit of tissue, or a portion of a pure culture of this organism from the body of an animal dead of this disease, results in a reproduction of the conditions found in the dead animal from which the tissues or cultures were obtained.

It sometimes happens that in guinea-pigs which have been inoculated with this organism local pus-formations result, instead of a general septicemia. The organisms will then be found in the pus-cavity.

**BACTERIUM INFLUENZÆ (R. PFEIFFER), LEHMANN
AND NEUMANN, 1896.**

SYNONYM: Influenza bacillus, R. Pfeiffer, 1892.

An important historic epidemic disease, on the nature of which much light has been shed through modern methods

of investigation, is influenza. Quoting Hirsch:—the first trustworthy literary records that we have of this disease date from the early part of the twelfth century.

Between 1173 and 1874 it made its epidemic or pandemic appearance on eighty-six different occasions. Its first appearance in this country was in Massachusetts in 1672; since that time there have been twenty-two visitations of influenza in the United States. The pandemic of 1889–90, the most severe for a long time, appears to have originated in Central Asia and to have spread pretty much over the entire civilized world. The advent of influenza in a community is always remarkable for its astonishing rate of transmission from person to person and its dissemination over wide areas.

During the recent pandemic investigations having for their object the discovery of its cause were instituted, with the result of demonstrating in the catarrhal secretions from the air-passages a micro-organism that is claimed to stand in causal relation to influenza.

By appropriate methods of staining it is also frequently possible to demonstrate the presence of this organism in the secretions of the nose, mouth, and throat of apparently healthy persons, as well as in those from persons suffering from such diseases as diphtheria, scarlet fever, measles, etc.

This organism, *bacterium influenzæ*, as it is called, was discovered, isolated, cultivated, and described by R. Pfeiffer.

It is a very small, slender, non-spore-forming, non-motile, aërobic bacillus, occurring singly and in pairs, joined end to end. It stains with watery solutions of the ordinary basic aniline dyes; somewhat better with alkaline methylene-blue, but best when treated for five minutes with a dilution of Ziehl's carbol-fuchsin in water (the color of the solution

should be pale red). (Fig. 76.) It is decolorized by the method of Gram.

It develops only at temperatures ranging from 26° to 43° C. Its optimum temperature for growth is 37° C. It possesses the peculiarity of developing upon only those artificial culture-media to which blood or blood-coloring-matter has been added. Its cultivation is best conducted

FIG. 76



Bacterium influenzae in sputum.

and its development most satisfactorily observed by the following procedure: over the surface of a slanted agar tube or over agar-agar solidified in a Petri dish smear a small quantity of sterile blood (not blood-serum). A bit of the mucus from the sputum of the influenza patient is then taken up with sterilized forceps or on a sterilized wire loop, rinsed in sterile bouillon or water and rubbed over the surface of the prepared agar-agar. The plate or tube is then

placed in the incubator at 37° to 38° C. If influenza bacilli be present, they will develop as minute, transparent, watery colonies that are without structure, and which resemble somewhat minute drops of dew. They are discrete and show little or no tendency to coalesce.

If a small bit of mucus be rubbed over the surface of ordinary nutrient agar-agar, no such colonies develop. In making the diagnosis by this method cultures on both agar-agar containing blood (not blood-serum) and agar-agar containing no blood should always be made, for the reason that growth of these peculiar colonies in the former and no such growth in the latter are evidence that one is dealing with materials from a case of influenza.

The organism may also be cultivated in bouillon to which blood has been added, if kept at body-temperature. The growth appears as whitish flakes. Since this organism is a strict aërobe, its cultivation can only be conducted on the surface of the medium used—*i. e.*, where it has freest access to oxygen. It is therefore inadvisable to prepare plates in the usual way. When its cultivation is attempted in bouillon it is recommended, in order to favor the free diffusion of oxygen, that the depth of fluid be very shallow.

Contrary to what might be supposed, *bacterium influenzae* has very little tenacity of life outside of the diseased body. It is destroyed in from two to three hours by rapid drying, and in from eight to twenty-four hours when dried more slowly. Cultures retain their vitality for from two to three weeks. The organism dies in water in a little over a day. As a result of these observations, Pfeiffer does not believe the disease to be disseminated by either the air or the water, but rather by direct infection from the catarrhal secretions of the patients.

This organism has not been found outside of the human body. In the influenza patient it is present in the catarrhal secretions, bronchial mucous membrane, and the diseased lung-tissues. It may be demonstrated microscopically in the mucus by cover-slip preparations made in the usual way and stained with diluted carbol-fuchsin, referred to above. In the tissues it may be demonstrated in sections stained in the same solution. In the sputum the bacteria are found as masses and as scattered cells. (See Fig. 76.) They are also found within the bodies of leukocytes, especially in the later stages of the disease when convalescence has set in; at this time they appear as very small, irregular, evidently degenerated bacteria within white blood-corpuscles. They are also present in the nasal secretions.

At autopsies it is advisable to cut out pieces of the diseased tissue about the size of a pea or a bean, break them up in a small quantity of sterile water or bouillon, and make the cultures from this infusion. By this procedure two advantages are gained: first, a dilution of the number of bacteria present; and, secondly, the tissue furnishes the amount of hemoglobin necessary for the growth of the organism. Under these circumstances it is, of course, not necessary to make a further addition of blood to the culture-medium.

The only animal that has been found susceptible to inoculation with this organism is the monkey. By intratracheal injection Pfeiffer succeeded in causing a toxic condition that proved fatal. He does not regard the death of the animals as due to general infection, but rather to intoxication. The disease, as seen in man, has not been reproduced in animals.

CHAPTER XXI.

Tuberculosis—Microscopic Appearance of Miliary Tubercles—Diffuse Caseation—Cavity-formation—Encapsulation of Tuberculous Foci—Primary Infection—Modes of Infection—The Bacterium Tuberculosis—Location of the Bacilli in the Tissues—Microscopic Appearance of Bacterium Tuberculosis—Staining Peculiarities—Organisms with which Bacterium Tuberculosis may be Confounded: Bacterium Lepræ; Bacterium Smegmatis—Acid-proof Bacteria—Bacterium Tuberculosis Avium—Variations—Pseudotuberculosis—Susceptibility of Animals—Tuberculin—Vaccination Against Tuberculosis—Actinomyces Bovis—Actinomyces Israëlī, Actinomyces Maduræ, Actinomyces Farcinicus, Actinomyces Eppingeri, Actinomyces Pseudotuberculosis.

BACTERIUM TUBERCULOSIS (KOCH), MIGULA, 1900.

SYNONYM: *Bacillus tuberculosis*, Koch, 1882.

LOCAL OR GENERAL TUBERCULOSIS.—Should the animal succumb to neither of the infections just described, then its death from tuberculosis may reasonably be expected.

When this disease is in progress alterations in the lymphatic glands nearest the site of inoculation may be detected by the touch in from two to four weeks. They will then be found enlarged. Though not constant, tumefaction and subsequent ulceration at the point of inoculation may be observed. Progressive emaciation, loss of appetite, and difficulty in respiration point to the existence of the general tuberculous process. Death ensues in from four to eight weeks after inoculation. At autopsy either general or local tuberculosis may be found. The expressions of tuberculosis are so manifold and in different animals vary so widely the

one from the other, that no fixed law as to what will appear at autopsy can *à priori* be laid down.

The guinea-pig, which is best suited for this experiment because its susceptibility to tuberculosis is greater and more constant than that of other animals usually found in the laboratory, presents, in the main, changes that are characterized by coagulation-necrosis and caseation. This is particularly the case when the infection is general—*i. e.*, when the process is of the acute miliary type; then the tissues of the liver and spleen present the most favorable field for the study of this pathological-anatomical alteration.

In general, the tubercular lesions can be divided into those of strictly focal character—*i. e.*, the miliary and the conglomerate tubercles—and those which are more diffuse. The latter lesions, although primarily of the same nature as the miliary tubercles, are much greater in extent and not so sharply circumscribed. These latter lesions play a more conspicuous *rôle* in the pathology of the disease than do the miliary nodules, although it is the miliary nodules (tubercles) that give to the disease its name.

At autopsy the pathological manifestations of the disease are not infrequently seen to be confined to the seat of inoculation and to the neighboring lymphatic glands. These tissues then present all the characteristics of the tuberculous process in the stage of cheesy degeneration. When the disease is more general the degree of its extension varies. Sometimes the small gray nodules—miliary tubercles—are only to be seen with the naked eye in the tissues of the liver and spleen. Again, they may invade the lung, and frequently they are distributed over the serous membranes of the intestines, the lungs, the heart, and the brain. These gray nodules, as seen by the naked eye, vary in size from that of

a pin-point to that of a hempseed, and, as a rule, are, in this stage, the result of the fusion of two or more still smaller foci. Though the two terms "miliary" and "conglomerate" are employed for the description of the macroscopic appearances of these nodules, yet it is very rarely that any condition other than that due to the fusion of several of these minute foci can be detected by the naked eye.

The miliary tubercles are of a pale gray color, with a white centre, are slightly elevated above the surface of the tissue in which they are located, and, as stated, vary considerably in dimensions, usually appearing as points which range in size from that of a pin-point to that of a pin-head. They are not only located upon the surface of the organs, but are distributed through the depths of the tissues. To the touch they sometimes present nothing characteristic, but when closely packed together in large numbers they usually give a mealy or sandy sensation to the hand passed over them. Stained sections of miliary tubercles present a distinctly characteristic appearance, and the disease may be recognized by these histological changes alone, though the crucial test in the diagnosis is the demonstration of tubercle bacilli within the nodules.

Microscopic Appearance of Miliary Tubercles.—A miliary tubercule under a low magnifying power of the microscope presents somewhat the following appearance: there is a central pale area, evidently composed of necrotic tissue because of its incapacity for taking up the nuclear stains commonly employed. Scattered through this necrotic area may be seen granular masses irregular in size and shape; they take up the stains employed and are evidently fragments of cell-nuclei in course of destruction. Throughout the necrotic area may be seen irregular lines, bands, or

ridges, the remains of tissues not yet completely destroyed. Around the periphery of this area may sometimes be noticed large multinucleated cells, the nuclei of which are arranged about the periphery of the cell or grouped irregularly at its poles. The arrangement of these nuclei as observed in sections is usually oval, or somewhat crescentic. In tubercles from the human subject these large "giant-cells," as they are called, are quite common. They are much less frequent in tubercular tissues from lower animals.

Round about the central focus of necrosis is seen a more or less broad zone of closely packed small round and oval bodies, which stain readily but not homogeneously. They vary in size and shape, and are seen to be imbedded in a delicate network of fibrinous-looking tissue. This fibrin-like network in which these bodies lie, and which is a common accompaniment of giant-cell formation, is in part composed of fibrin, but is in the main, most probably, the remains of the interstitial fibrous tissue of the part. This zone of which we are speaking is the zone of so-called "granulation-tissue," and consists of leukocytes, granulation-cells, fibrin, and the fibrous remains of the organ; the irregularly oval, granular bodies which take up the stain are the nuclei of these cells. The zone of granulation-tissue surrounds the whole of the tuberculous process, and at its periphery may fade gradually into the healthy surrounding tissues or be sharply outlined or may fuse with a similar zone surrounding another tubercular focus.

Diffuse Caseation.—The diffuse caseation, as said, plays a more important *rôle* in the tuberculous lesion, both in the human and experimental forms, than does the formation of miliary tubercles. Here a large area of tissue undergoes the same process of necrosis and caseation as the centre of

the miliary tubercle. In certain tissues, notably the lungs and lymphatics, it is more marked than in others. In rabbits, particularly, all the changes in the lung frequently come under this head. When this is the case solid masses are found, sometimes as large as a pea, or involving even an entire lobe or the whole lung in some cases. They are opaque and of a whitish-yellow color, and on section are peculiarly dry and hard. Entire lymphatic glands may be changed in this way. The conditions which appear to be most favorable to the occurrence of this widespread caseation of the tissues are the simultaneous deposition of masses of tubercle bacilli in them, and the involvement of a wide area instead of a single isolated point, as in the miliary tubercle. Necrosis is so rapid that time does not suffice for those reactive changes to take place in the tissues which result in the formation of the outer zone of the miliary tubercle. In other instances the entire caseous area is surrounded by a granulation-zone similar to that around the caseous centre of the miliary tubercles. It is of special importance to recognize the etiological connection between this diffuse caseation and the tubercle bacillus, because until its nature was accurately determined caseous pneumonia of the lungs formed the chief obstacle which many encountered in recognizing the specific infectiousness of tuberculosis.

Cavity-formation.—The production of cavities, a prominent feature in human tuberculosis, particularly of the lungs, is due to softening of the necrotic, caseous masses or of aggregations of miliary tubercles. The material softens, is expelled by way of the bronchi, and a cavity results. In the wall of this cavity the tuberculous changes still proceed, both as diffuse caseation and formation of miliary

tubercles. The whole cavity with the reactive changes in the tissues of its walls may be properly conceived as a single gigantic tubercle, its wall forming a tissue very analogous to the outer zone of the single tubercle, the cavity itself corresponding to the caseous centre.

In animals used for experiment cavity-formation of this sort is very rare, owing to the greater resistance of the caseous tissue. That it is, however, possible to produce in rabbits conditions that eventuate in pulmonary cavities in all physical respects similar to those seen in the human being has been beautifully demonstrated by Prudden. He showed that when he had injected fluid cultures of *streptococcus pyogenes* into the trachea of rabbits already affected with tubercular consolidation of the lungs, the result of the mixed infection thus brought about was cavity-formation in eight out of nine lungs subjected to the conditions of the experiment; while in only one out of eleven did cavities form under the influence of the tubercle bacillus alone.¹ The investigations of Ayer² not only confirm the findings of Prudden, but reveal additional facts of very great practical importance. He demonstrates that experimental cavity formation is very largely dependent upon the mass, physically speaking, of tubercle bacilli used for the intratracheal injection; that uncomplicated tubercular infection is not usually accompanied by fever, but that if there be engrafted upon such infection, another type of infection (in Ayer's Experiments, Streptococcus Infection) that fever was observed in something over 69 per cent. of the animals used in his investigations.

¹ Prudden, Experimental Phthisis in Rabbits, with the Formation of Cavities, etc., Transactions of the Association of American Physicians, 1894, ix, 166.

² Journal of Medical Research, November 2, 1914, xxx, 141.

In the contents and in the walls of tubercular cavities in man bacteria other than *bacillus tuberculosis* are found. It is to the influence of some of these, as we have seen, that diseases other than tuberculosis may sometimes be produced by the inoculation of animals with the sputum from such cases; and it is also to the absorption of their toxic products that some of the constitutional manifestations, particularly fever, commonly seen in cases of advanced pulmonary tuberculosis are attributed.

Encapsulation of Tubercular Foci.—It not uncommonly occurs that round about a necrotic tuberculous focus there is formed a fibrous capsule which may completely shut off the diseased from the healthy tissue surrounding it; or a tuberculous focus may, through the resistance of the tissue in which it is located, be more or less completely isolated. In this condition the diseased foci may lie dormant for a long time and give no evidence of their existence, until they are made to break through their envelopes by some disturbing cause. With the passage of the bacilli from such a focus into the vascular or lymphatic circulation the disease may become general.

It is to some such accident as this that the sudden appearance of general tubercular infection in subjects supposed to have recovered from the primary local manifestations may often be attributed. The breaking-down of old caseous lymphatic glands is a common example of this recurrence of tuberculosis.

Primary Infection.—Primary infection occurs through either the vascular or lymphatic circulation. Through these channels the bacilli gain access to the tissues and become lodged in the finer capillary ramifications or in the more minute lymph-spaces. Here they find conditions

favorable to their development, and in the course of their life-processes produce substances of a chemical nature which serve to bring about characteristic changes in the immediate neighborhood. In the beginning the fixed cells, particularly the endothelial cells of the capillaries and lymph-spaces, are stimulated to proliferation. With the onset of this phenomenon, evidence of other cell multiplication may readily be detected in and about the affected focus. As proliferation continues and as the local circulation becomes more and more impaired, the centre of the diseased area gradually assumes a condition of inactivity, and ultimately presents all the characteristics of dead and dying tissue. This death of tissue is one of the earliest, the most easily recognized, and the most characteristic results of tubercular infection, and may usually be detected, in greater or less degree, even in the youngest and most minute tubercles. With the production of this progressive necrosis—for progressive it is, as it proceeds as long as the bacilli live and continue to produce their poisonous products—there is in addition a reactive change in the surrounding tissues, which results in the formation of a granulation-zone at the outer margins of the dying and dead tissue. This zone consists of small, round granulation-cells and of leukocytes, all of which are seen in the meshes of the finer fibrous tissues of the part. At the same time alterations are produced in the walls of the vessels of the locality; these tend to occlude them, and thus the process of tissue-death is favored by a diminution of the amount of nutrition brought to them. These changes may continue until eventually conglomerate tubercles, widespread caseation, or cavity-formation results; or from one cause or another the life-processes of the bacilli may be checked and recovery occur.

Modes of Infection.—Experimentally, tuberculosis may be produced in susceptible animals by subcutaneous inoculation, by direct injection into the circulation, by injection into the peritoneal cavity, by feeding of tuberculous material, by the introduction of the bacilli into the air-passages, and by inoculation into the anterior chamber of the eye.

In the human subject the most common portals of infection are, doubtless, the air-passages, the alimentary tract, and cutaneous wounds. When introduced subcutaneously the resulting process finds its most pronounced expression in the lymphatic system. The growing bacilli make their way into the lymphatic spaces of the loose cellular tissue, are taken up in the lymph-stream and deposited in the neighboring lymphatic glands. Here they may remain and give rise to no alteration other than that seen in the glands themselves; or they may pass on to neighboring glands, and eventually be disseminated throughout the lymphatic system, ultimately reaching the vascular system.

Having gained access to the bloodvessels the results are the same as those following intravascular injection of the bacilli, namely, general tuberculosis quickly follows, with the production of miliary tubercles most conspicuous in the lungs and kidneys; less numerous in the spleen, liver, and bone-marrow.

When inhaled into the lungs, if conditions are favorable, multiplication of the bacilli quickly occurs. Coincident with their growth they are mechanically pressed into the tissues of the lungs. As multiplication continues some are transported from the primary site of infection to healthy portions of the lung-tissue, where, through their development, the process is repeated.

In the same way infection by way of the alimentary tract

is in the main due to the bacilli being forced by mechanical pressure into the walls of the intestines. Investigation has shown that lesions of the intestinal coats are not necessary for the entrance of tubercle bacilli from the lumen of the gut into the internal organs and tissues. They may be transported from the intestinal tract into the lymphatics in the same way that the fat-droplets of the chyle find entrance into the lymphatic circulation.

They may gain access to the tissues by way of the tonsils.

Unlike most pathogenic organisms, the tubercle bacillus is resistant to drying. When thrown off from the lungs in the sputum of tuberculous patients, unless special precautions be taken to prevent it, the sputum becomes dried, is ground into dust, and sets free in the atmosphere the tubercle bacilli which came with it from the lungs, and which have the property of exciting the disease in susceptible persons who inhale them. The greater frequency of *pulmonary* tuberculosis over other manifestations of the disease, points to this as one of the commonest sources and modes of infection in human beings. This opinion is borne out both by statistical studies upon the disease and by the fact that the dust collected from apartments occupied by tubercular patients is often contaminated with living tubercle bacilli.¹

Location of the Bacilli in the Tissues.—The bacilli will be found most numerous in those tissues in which the disease is most active.

In the initial stage of the disease the bacilli will be fewer in number than later; at this time only scattered bacilli may be found; later they are more numerous; and, finally,

¹ Cornet, Zeitschrift für Hygiene, 1889, Bd. v, S. 191.

when the process has advanced to a stage easily recognizable by the naked eye, they are distributed through the granulation-zones in clumps and scattered about in large numbers.

In the central necrotic masses, which consist of cell-detritus, it is rare that the organisms can be demonstrated microscopically: It is at the periphery of these areas and in the progressing granular zone that they are to be seen most frequently.

This apparent absence of the bacilli from the central necrotic area and often from old caseous tissues must not be taken, however, as evidence that these materials are not infective, for with them the disease can be reproduced in susceptible animals by inoculation. A conspicuous example of this condition is seen in old scrofulous glands. These glands usually present a slow process, are commonly caseous, and always possess the property of producing the disease when introduced into the tissues of susceptible animals, but yet they are the most difficult of all tissues in which to demonstrate microscopically the presence of tubercle bacilli.

In tubercles containing giant-cells the bacilli can usually be demonstrated in the granular contents of these cells. Frequently they will be found accumulated at the pole of the cell opposite to that occupied by the nuclei, as if there existed an antagonism between the nuclei and the bacilli. In some of these cells, however, the distribution of the bacilli is seen to be irregular, and they will be found scattered among the nuclei as well as in the necrotic centre of the cell. As the number of bacilli in the giant-cell increases the cell itself is ultimately destroyed.

Tubercular tissues always contain the bacilli and are always capable of reproducing the disease when introduced

into the body of a susceptible animal. From the tissues of this animal the bacilli may be obtained and cultivated artificially, and these cultures are capable of again producing the disease when further inoculated. Thus are fulfilled the postulates formulated by Koch for proving the etiological rôle of an organism in the production of a malady.

THE BACTERIUM TUBERCULOSIS.

Of the three pathogenic organisms liable to occur in the sputum of a tuberculous subject, the tubercle bacillus gives us the greatest difficulty in our efforts at cultivation.

It is almost an obligate parasite, and finds conditions entirely favorable to its development only in the animal body. On ordinary artificial media the bacilli taken directly from the animal body grow only very imperfectly, or, in many cases, not at all. From this it seems probable that there is a difference in the nature of individual tubercle bacilli—some appearing to be capable of growth in the animal tissues only, while others are apparently possessed of the power to lead a limited saprophytic existence. It may be, therefore, that those bacilli which we obtain as artificial cultures from the animal body are offsprings of the more saprophytic varieties. At best, one never sees with the tubercle bacillus a saprophytic condition in any degree comparable to that possessed by many of the other organisms with which we have to deal.

For the cultivation of bacillus tuberculosis directly from the tissues of the animal, the best method is that recommended by Koch, viz., cultivation upon blood-serum. Its parastitic tendencies are so pronounced that even very slight variations in the conditions under which one endeavors

to isolate bacillus tuberculosis from the tissues may cause total failure. It is, therefore, necessary that the injunctions for obtaining it in pure culture be carefully observed.

Preparation of Cultures from Tissues.—Under strict aseptic precautions remove from the animal a diseased organ—the liver, spleen, or a lymphatic gland being preferable. Place the tissue in a sterilized Petri dish, and dissect out with sterilized scissors and forceps the small tubercular nodules. Place each nodule upon the surface of the blood-serum, one nodule in each tube, and without attempting to break it up or smear it over the surface, leave it for four or five days in the incubator. After this time it may be rubbed over the surface of the serum. The object of this is to give to bacilli in the nodule an opportunity to multiply, under the favorable conditions of temperature and moisture, before an effort is made to distribute them over the surface of the medium. It is best to dissect away twenty to thirty such tubercles and treat each in the same way. Some of the tubes will remain sterile, others may be contaminated by extraneous saprophytic organisms during the manipulation, while a few may give the result desired, viz., a growth of the tubercle bacillus itself.

The blood-serum upon which the organism is to be cultivated should be comparatively freshly prepared—that is, should not be dry.

After inoculating the tubes they should be carefully sealed to prevent evaporation and consequent drying. This is done by burning off the overhanging cotton plug in a gas-flame, and then impregnating the upper layers of the cotton with either sealing-wax or paraffin of a high melting-point; or by inserting over the burned end of the cotton plug a soft, closely fitting cork that has been sterilized

in the steam sterilizer just before using (Ghriskey). This precaution is necessary because under the most favorable conditions tubercle bacilli directly from the animal body show no evidence of growth for about twelve days after inoculation upon blood-serum, and, as they must be retained during this time at the body-temperature— 37.5° C.—evaporation would take place very rapidly and the medium would become too dry for their development.

If these primary efforts result in a growth of the bacilli, further cultivations may be made by taking up a bit of the colony, preferably a moderately large quantity, and transferring it to fresh serum, and this in turn is sealed up and retained at body temperature. Once having obtained the organism in pure culture, its subsequent cultivation may be conducted upon the glycerin-agar-agar mixture—ordinary neutral nutrient agar-agar to which from 4 to 6 per cent. of glycerin has been added. This is a very favorable medium for the growth of this organism after it has accommodated itself to its saprophytic mode of existence, though blood-serum is perhaps the best medium to be employed in obtaining the first generation of the organism from tuberculous tissues.

The organism may be cultivated also on neutral milk to which 1 per cent. of agar-agar has been added, also upon the surface of potato, and likewise in meat-infusion bouillon containing from 4 to 6 per cent. of glycerin.

Cultures of the tubercle bacillus are characteristic in appearance—once having seen them there is little probability of subsequent mistake. They appear as dry masses, which may develop upon the surface of the medium either as flat scales or as coarse, heaped up, granular masses. They are never moist, and frequently have the appearance

of dry meal spread upon the surface of the medium. In the lower part of the tube in which they are growing—*i. e.*, that part occupied by a few drops of fluid which has in part been squeezed from the medium during the process of solidification, and is in part water of condensation—the colonies may be seen to float as a thin pellicle upon the surface of the fluid.

The individuals composing the growth adhere so tenaciously together that it is with the greatest difficulty they can be separated. In even the oldest and dryest cultures pulverization is impossible. The masses can only be separated and broken up by grinding in a mortar with the addition of some foreign substance, such as very fine, sterilized sand, or ground glass, etc.

The cultures are of a dirty-drab or brownish-gray color when seen on serum or glycerin-agar-agar.

On potato they grow in practically the same way, though the development is much more limited. On this medium they are of nearly the same color as the potato on which they are growing. When cultivated for a time on potato they are said to lose their pathogenic properties.

On milk-agar-agar they are of so nearly the same color as the medium that, unless they are growing as characteristic mealy-looking masses, considerably elevated above the surface, their presence is less conspicuous than when on other media.

In bouillon they grow as a thin pellicle on the surface. This may fall to the bottom of the fluid and continue to develop, its place on the surface being taken by a second pellicle.

The tubercle bacillus does not develop on gelatin because of the low temperature at which this medium must be used.

Microscopic Appearance of Bacterium Tuberculosis.—Microscopically the organism itself is a delicate rod, usually somewhat beaded in its structure, though rarely it is seen to be homogeneous. It is either quite straight, or somewhat curved or bent on its long axis. In some preparations involution-forms, consisting of rods a little clubbed at one extremity or slightly bulging at different points, may be detected. Branching forms of this organism have been described. It varies in length—sometimes being seen in very short segments, again much longer, though never as long threads. Usually its length varies from 2 to 5 μ . It is commonly described as being in length about one-fourth to one-half the diameter of a red blood-corpuscle. It is very slender. (See Fig. 74.)

These rods usually present, as has been said, an appearance of alternate stained and colorless portions. At times these colorless portions are seen to bulge slightly beyond the contour of the rod, and in this way give to the rods the beaded appearance so commonly ascribed to them. These oval colorless areas were at one time thought to be spores. A number of competent observers have expressed the opinion that the rods which we see in tubercular lesions and which we call bacillus tuberculosis are not, strictly speaking, bacilli, but are fragments or developmental phases of a more highly organized fungus—possibly related to the streptothrices or actinomyces. The point cannot now be decided.

Staining Peculiarities.—A peculiarity of this organism is its behavior toward staining-reagents, and by this means alone it may easily be recognized. The tubercle bacillus does not stain by the ordinary methods. It possesses a peculiarity in its composition that renders it proof against the simpler staining processes. It is therefore necessary

that more energetic and penetrating reagents than the ordinary watery solutions be employed. Experience has taught us that certain substances not only increase the solubility of the aniline dyes, but their penetration as well. Two of these are aniline oil and carbolic acid. They are employed in the solutions to about the point of saturation. (For the methods of staining *B. tuberculosis* see Chapter on Staining.)

Under the influence of heat these solutions are seen to stain all bacteria very intensely—the tubercle bacilli as well as other forms. If we subject our preparation, which may contain a mixture of tubercle bacilli and other species, to the action of decolorizing-agents, another peculiarity of the tubercle bacillus will be observed. While all other organisms in the preparation give up their color and become invisible, the tubercle bacillus retains it with marked tenacity. It stains with great difficulty; but once stained it retains the color even under the action of strong decolorizing-agents.

Variations of *B. Tuberculosis*.—Theobald Smith¹ has called attention to certain very conspicuous differences that may be observed between the bacilli obtained from human and those from bovine tuberculosis; and in a series of inoculation experiments Ravenel has shown that for a large number of animal species tubercle bacilli of bovine origin were constantly more virulent than those from human sources.

Susceptibility of Animals to Tuberculosis.—The animals that are known to be susceptible to tuberculosis are man, apes, cattle, horses, sheep, hogs, guinea-pigs, pigeons, rabbits, cats, and field-mice. White mice, dogs, and rats possess immunity from the disease.

¹ Transactions of the Association of American Physicians, 1896, xi, 275.

Tuberculin.—The filtered sterile products of growth from old fluid cultures of the tubercle bacillus represent what is known as *tuberculin*—a solution containing a group of protein substances possessing most interesting properties. When injected subcutaneously into healthy subjects tuberculin has no effect; but when introduced into the body of a tuberculous person or animal a pronounced systemic reaction results, consisting of sudden but temporary elevation of temperature, with, at the same time, the occurrence of marked hyperemia about the tuberculous focus, a change histologically analogous to that seen in the primary stages of acute inflammation. This zone of hyperemia, with the coincident exudation and infiltration of cellular elements, probably aids in the isolation or casting off of the tuberculous nodule, the inflammatory zone forming, so to speak, a line of demarcation between the diseased and healthy tissue.

As a curative agent for tuberculosis, tuberculin has not proved worthy of the confidence that was at first accorded to it. Its field of usefulness is now almost limited to the diagnosis of obscure cases.

In veterinary medicine it has proved trustworthy as a diagnostic aid, and is practically everywhere in use for the detection of incipient tuberculosis in cattle.

VACCINATION AGAINST TUBERCULOSIS.—Experiments by Pearson and Gilliland, v. Behring, and others have shown that it is possible to partly immunize animals with lowly virulent tubercle bacteria of human origin. After one or two injections with such organisms the animals showed for a time some degree of tolerance to the more highly virulent bovine strains. The results of experiments in this direction have been so encouraging as to justify further research

in this direction, but complete immunity has not as yet resulted.

We have reviewed the three common pathogenic organisms that may be encountered in the sputum of tuberculous individuals. Occasionally other species may be present. The pyogenic forms are not rarely found, and for some time after an attack of diphtheria the bacillus of Löffler is demonstrable in the pharynx, so that it, too, may be present under exceptional circumstances.

Organisms with which *Bacterium Tuberculosis* may be Confused.—It is important to note that in the study of tuberculosis one may fall into error unless it be borne in mind that there is a group of micro-organisms whose members are in many respects so like the genuine bacillus tuberculosis as easily to be mistaken for it. While its peculiar micro-chemical reaction is usually sufficient for identification, particularly in connection with human pathological lesions, it is well to remember that the confusing organisms are not only characterized by the same staining peculiarities as bacillus tuberculosis, but may readily be mistaken for it on morphological grounds also. Furthermore, while not all the members of this group are capable of causing disease, some of them are pathogenic for the same animals that are susceptible to true tubercular infection; and there may produce in those animals lesions which are distinguishable from genuine tubercles only by their finer histological structure. A few words concerning some of these varieties, with a brief summary of their more important peculiarities, may not be out of place.

BACTERIUM LEPRÆ.—Between 1879 and 1881 there was described by Hansen and by Neisser an organism, a bacillus,

that was constantly to be found in the nodules, characteristic of leprosy. For this organism the name *bacillus lepræ* was suggested. Though very like bacterium tuberculosis in both morphology and staining properties, it is, however, a little shorter, thicker, and much less homogeneously stained. Its presence in the tissues and secretions is demonstrated by the same method as that employed for detecting bacillus tuberculosis. In secretions of leprous nodules, stained by the ordinary Koch-Ehrlich process, the bacilli, crowded together in the large so-called "lepra cells," are always to be seen in great abundance. Numerous efforts to cultivate bacillus lepræ from the diseased tissues and to reproduce the disease by inoculation have led to little more than a mass of confusing results. It is possible that a recent observation of Johnston¹ may assist in clearing away some at least of the confusion. Johnston believes the acid-proof, so-called bacilli, seen in the lepra cells to be developmental phases of a streptothrix which is itself *not* acid proof. His opinion appears to be justified by the results of carefully made culture and inoculation studies.²

BACTERIUM SMEGMATIS.—In 1885 Alvarez and Tavel discovered in the fatty secretions about the genitalia an organism that suggested the bacterium of tuberculosis. Their observation has been abundantly confirmed by others, and the organism to which they directed attention is now regarded as pretty commonly present in the smegma. It is known, therefore, as the smegma bacterium (*bacterium smegmatis*). In this secretion it is found in clumps located upon or within epithelial cells. It stains by the method

¹ Philippine Journal of Science, June, 1914, vol. ix, No. 3, Section B, Tropical Medicine, p. 227.

² For a general discussion on this subject, together with literary references see Wolbach and Honeij, Journal of Medical Research, 1914, xxix, 367.

used in staining bacterium tuberculosis. It has no pathogenic power. It is said to have been artificially cultivated upon coagulated hydrocele fluid and in milk.

THE ACID-PROOF BACTERIA.—In addition to the species mentioned, quite a group of other “acid-proof” bacteria, as they are called, have been described by different investigators. They are characterized by staining, as does bacterium tuberculosis, by retaining the stain to a greater or less extent when treated with acids and alcohol, and by being in many instances strikingly like bacterium tuberculosis in their morphology. The members of this group seem to be distributed pretty widely in nature. They have been detected in non-tuberculous sputum, in gangrene of the lung, in the normal intestinal contents of man and domestic animals, in certain of the cold-blooded species, in the soil, in fodder—*i. e.*, grass, hay and seed—in manure, and in butter. They are not regularly found under any of these conditions, and they are rarely present in very large numbers. Inasmuch as they are occasionally encountered under circumstances that might lead one to look for true tubercle bacilli, and since they possess certain peculiarities similar to those by which it has been the custom to identify bacillus tuberculosis—*i. e.*, retention of the stain when acted upon by acids or alcohol, and a more or less delicate, beaded form—the possibility of their being confounded with that organism is obvious. In consequence they have received a great deal of attention during the past few years.

Space does not permit of a description of the twenty odd species (?) that have been described by different investigators. It will suffice to say, from personal study of the group, that in all probability not more than three, per-

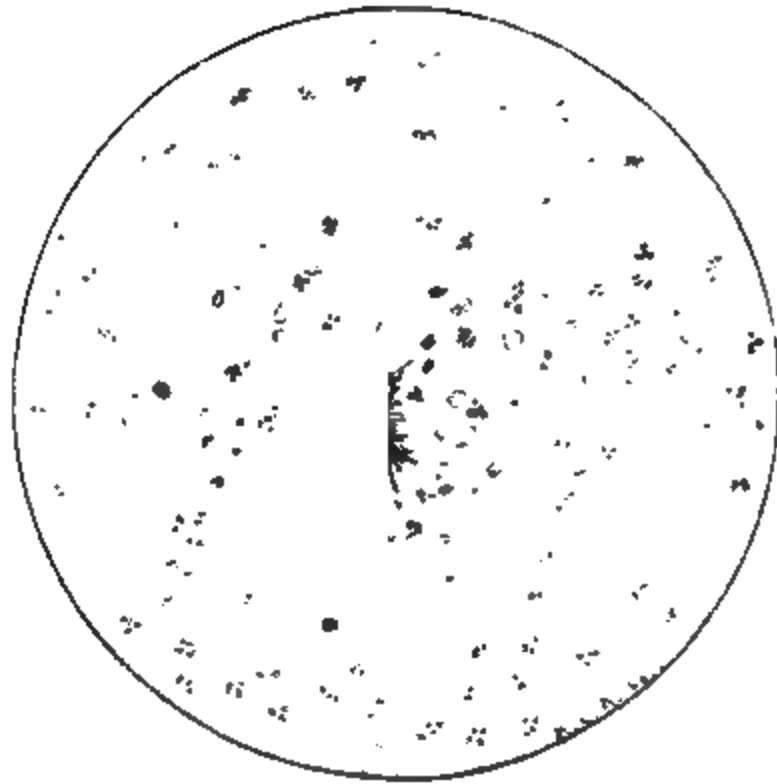
haps only two, species are really represented, and that the remainder may fairly be regarded as varieties. As said, the characteristic common to all the members of this group is that they are to greater or less extent acid-proof—*i. e.*, when once stained by the Koch-Ehrlich or Ziehl process the color is not in all cases removed by the ordinary acid decolorizers. In this particular, however, there is such a striking difference between the degree of their resistance to acid decolorizers and that of the tubercle bacillus as to render this an important differential aid; for instance, the tubercle bacillus, when stained, may be treated for several minutes with so strong a decolorizer as 30 per cent. nitric acid without losing its color; whereas, none of the members of this group retain their color after a few seconds of such treatment, and particularly if it be followed by washing in alcohol. In morphology some of them might readily be mistaken for bacillus tuberculosis, though even these are usually a trifle larger and less delicately formed than that organism; others are at once differentiated from normal tubercle bacilli, but have somewhat their appearance when degenerated or involuted; still others have nothing in their general appearance to lead to confusion.

When mixed with other bacteria, as is the case in the soil, in manure, in intestinal contents, etc., their isolation in pure culture is a matter of difficulty, and this is by no means lessened by the fact that under these circumstances they are always numerically in the minority. When present in butter, their isolation offers fewer difficulties, for by the injection of the butter containing them into the peritoneal cavity of guinea-pigs conditions are created that favor their development, and from animals so treated they may usually be recovered in pure culture.

When studied in pure culture, all of them are at once distinguished from bacillus tuberculosis by the following group characteristics: they are of relatively rapid growth, there being usually an abundant development on glycerin-agar-agar after twenty-four to forty-eight hours at body-temperature; they grow well but less rapidly at ordinary room-temperature—*i. e.*, at 18° to 20° C.; they grow well in litmus-milk, and, as a rule, produce alkali that causes the color to become a deep blue; the growth on agar-agar is dry, shrivelled, and wrinkled in appearance, and of a soft, mealy consistency in some cases (Möller's grass bacillus II, Rabinowitsch's butter bacillus, for instance), while in others it is more membranous, as in the case of Möller's timothy bacillus. We have never seen in these cultures the hard, coarse granules so common to cultures of bacillus tuberculosis; on glycerin-agar-agar some of them, namely, the timothy bacillus of Möller and its varieties, grow with a distinct orange color, while others, the grass bacillus II of Möller, the butter bacillus of Rabinowitsch, and their closely allied varieties, begin as a grayish-white deposit which may ultimately become of a pale or even distinct salmon color.

When pure cultures of them are injected into such animals as rabbits or guinea-pigs, some of them have no effect, and others cause lesions of more or less importance, the result being dependent upon the quantity employed and the mode of inoculation. By subcutaneous or intraperitoneal injection of pure cultures the result is usually negative. Occasionally the superficial lymphatic glands in the neighborhood of the site of inoculation may be inflamed and purulent. This we have seen only after subcutaneous inoculation. If pure cultures be injected into the peritoneal cavity along

Fig. 77



Showing Actinomyces Development of Bacillus Tuberculosis in Lung of Rabbit, thirty days after intravenous injection of suspension of the organism.

Fig 78



Showing Actinomyces Development of Acid-resisting Bacteria (Butter Bacillus of Rabinowitsch) in Kidney of Rabbit, following upon intravenous injection of suspension of the organism

with some sterile, irritating substance, such as sterilized butter, a widespread fibrinopurulent peritonitis is commonly the result.

When injected directly into the circulation of rabbits, the kidneys are almost uniformly affected, and in the majority of instances they are, singularly enough, the only organs in which lesions are to be detected. If, for instance, a cubic centimeter of a carefully prepared suspension in bouillon of, let us say, Möller's grass bacillus II, be injected into the circulation of a rabbit, and the animal be killed after twelve to fourteen days, the kidneys will be found marked by gray or yellowish points that range in size from that of a pin-point to that of a pin-head. They are sometimes very few in number, but in other cases both kidneys may be thickly studded with them. Often they are not elevated above the cortex of the organ, but in as many cases they are sharply defined, yellow in color, and stand up prominently from the cortical surface, being at the same time so adherent to the capsule that the removal of the latter tears them out bodily from the substance of the organ. In the very early stages of development these nodules are often difficult to distinguish from young tubercles, the reaction of the tissues being, as in the case of tubercles, characterized by proliferation of the fixed cells with little evidence of leukocytic invasion; later on, true giant-cell formation is recorded by some observers. We have not seen this. Clumps of endothelial nuclei or of lymphoid cells that remotely suggest the arrangement seen in giant cells are often encountered, but we have not regarded them as true giant cells. When fully developed, the nodule may present a mixed condition of caseation and suppuration. The conditions, as a whole, when advanced suggest a low grade

of inflammatory reaction. Occasionally nodules are encountered, especially in the kidney, that cannot be distinguished from tubercles. The bacilli are always to be found within the nodules; most frequently as single rods or clumps of rods, occasionally as rosette-like mycelia very suggestive of the characteristic growth of the actinomyces fungus in the tissues. This mode of development has also been observed with *bacillus tuberculosis*. (Figs. 77 and 78.)

It is important to note the difference between the results of intravenous inoculation of rabbits with *bacillus tuberculosis* and with the organisms under consideration. When *bacillus tuberculosis* is employed, the lungs, as well as the kidneys, are always involved, while with the grass bacillus II, the timothy bacillus, and the butter bacillus, involvement of the lungs, in our experiments, has been the exception rather than the rule.

Another point of interest is the lack of tendency on the part of the non-tuberculous process to progress or become disseminated.

That the members of this group are botanically related to *bacillus tuberculosis* there seems little room for doubt; but from personal study and from available evidence from other sources it appears unlikely that they are, except experimentally, concerned in disease-production or that they are of importance to either human or animal pathology.¹

In the microscopic examination, particularly of urine, of secretions from about the anus, rectum, and genitalia, and of butter, it is manifestly of importance to bear in mind the existence of this confusing group, for it is in such secretions and substances that its members are most often en-

¹ For the literature on "acid-proof" bacilli, see Cowie, *Journal of Experimental Medicine*, 1900, v, 205.

countered. The smegma bacillus and the butter bacillus are especially liable to lead one into error of diagnosis. This is less apt to be the case with the comparatively rare lepra bacillus.

**BACTERIUM TUBERCULOSIS AVIUM (MAFFUCCI),
MIGULA, 1900.**

SYNONYMS: *Bacillus tuberculosis avium*, Maffucci, 1891; *Mycobacterium tuberculosis avium*, Lehmann and Neumann, 1896.

From time to time fowls are known to suffer from a form of tuberculosis that in a number of ways suggests human or mammalian tuberculosis. The bacillus causing the disease, the so-called bacillus of fowl tuberculosis, *bacillus tuberculosis avium*, while simulating the genuine bacillus tuberculosis morphologically, differs from it both in cultural and pathogenic peculiarities. Thus, for instance, it develops into much longer and somewhat thinner threads; grows rapidly on media without glycerin or glucose; does not grow on potato; develops as well at from 42° to 43° C. as at 37° to 38° C.;¹ its virulence is not diminished by cultivation at 43° C.; development on artificial media begins in from six to eight days after inoculation; young cultures on solid media are whitish, soft, and moist, becoming yellowish and slimy with age; it is somewhat more resistant to drying and high temperatures than the bacillus of mammalian tuberculosis; the results of its pathogenic activities are almost always chronic, are rarely located in the lungs or intestines, but are especially frequent in the liver and spleen; the lesions are conspicuously rich in bacteria, do

¹ The normal body-temperature of fowls ranges between 41.5° and 42.5° C.

not show the central necrotic area that characterize the mammalian tubercle; the disease is transmissible from the hen to the embryo chick; the only susceptible mammal is the rabbit; the guinea-pig and dog are naturally immune; it has the same micro-chemical staining-reactions as mammalian bacillus tuberculosis; it has never been certainly detected in human tuberculosis.

Some are inclined to regard this organism as but a variety of genuine bacillus tuberculosis, and it is not unreasonable to believe that the sojourn of that organism in the body of a refractory animal, whose normal temperature is so high as that of the fowl, when not fatal to the organism, might result in striking modifications of certain of its biological functions. In fact, Nocard¹ has shown that if the genuine bacillus tuberculosis from man be left in the peritoneal cavity of chickens (by the collodion-sac method of Metchnikoff, Roux, and Sallemбини, which see) for from five to eight months, they will, by the end of this time, have become so modified in their biological peculiarities as to simulate very closely the bacillus of fowl tuberculosis.

Moore² reports studies on bacterium tuberculosis avium in an epidemic occurring in California. He obtained pure cultures by inoculating glycerin-agar or blood-serum tubes directly from tuberculous livers and spleens. In the original cultures little difficulty was experienced in cultivating the organism on glycerin-agar, fresh dog-serum, Dorset's egg-medium, potato, and glycerin-bouillon. The general cultural peculiarities observed agreed with those described by Maffucci, Nocard, Straus and Gamaleia, and others. He states that the avian tubercle bacteria as found in the

¹ Annales de l'Institut Pasteur, 1898, p. 561.

² Journal of Medical Research, 1904, vol. vi.

tissues of the fowl resemble quite closely those of the bovine and human varieties in their size and general morphology. The average length of a large number of measurements was 2.7 microns. Moore also tested the pathogenesis of the freshly isolated avian tubercle bacteria on fowls, rabbits, guinea-pigs, and pigeons. The results of these inoculations, however, were unsatisfactory, as were also feeding experiments of healthy fowls with human tuberculous sputum rich in bacteria.

Pseudotuberculosis.—Anatomical lesions very suggestive of, though not identical with, those produced by bacillus tuberculosis, have also from time to time been observed in mice, rats, guinea-pigs, rabbits, cats, goats, bovines, hogs, and man. They do not appear to be of a specific nature as regards etiology, for the reason that different authors have described different organisms as the causative agents. These affections are usually classed under the name pseudotuberculosis.

ACTINOMYCETES.

The term actinomycetes is restricted to a group of organisms having morphological affinities with the bacteria on the one hand and the hyphomycetes on the other. They resemble the bacteria in that they occur as homogeneous threads which under artificial cultivation may become segmented into short bacillus- or coccus-like fragments. Furthermore, they are unlike the molds in that they have not a double wall; are not filled with fluid containing granules, and the segments are not separated from one another by a distinct partition. They simulate the molds in that they develop from spores into dichotomously branching threads, which ultimately form colonies having more or

less resemblance to true mycelia. Certain of the threads composing such a mycelium become fruit hyphæ, breaking up into round, glistening, spore-like bodies. As a rule, these spores are devoid of the high resistance to heat exhibited by bacterial spores, and are stainable by the ordinary methods.

The limits of this group are ill defined and its recognized components are not as a whole well understood.

The longest known and most carefully studied actinomycetes are *act. bovis*, *act. maduræ*, *act. farcinicus*, and *act. Eppingeri*, although many other varieties have been encountered in association with important and interesting pathological lesions.

The fact that certain bacteria, viz., *B. tuberculosis*, *B. mallei*, *B. diphtheriæ* are, as a rule, segmented and occasionally show a tendency to branch, has led to their being classified at times with the actinomycetes. On this point, however, there is as yet no concensus of opinion.

It is interesting to note that the pathological lesions in which actinomycetes have been detected show in many cases certain similarities to true tubercular processes, and in few instances, save for the absence of tubercle bacteria, as we usually see them, were indistinguishable from tuberculosis.

More or less imperfectly studied varieties of actinomycetes have been encountered in abscess of the brain, cerebrospinal meningitis, endocarditis, bronchopneumonia, pleuropneumonia, pustular exanthemata, abscess of the lung, bronchiectasis, pulmonary gangrene, necrosis of the vertebræ, subphrenic abscess, noma, and pseudotuberculosis.

In some cases the actinomycetes can be obtained in culture from the diseased tissues; almost as often they can not. Sometimes the inoculation of animals with bits of the

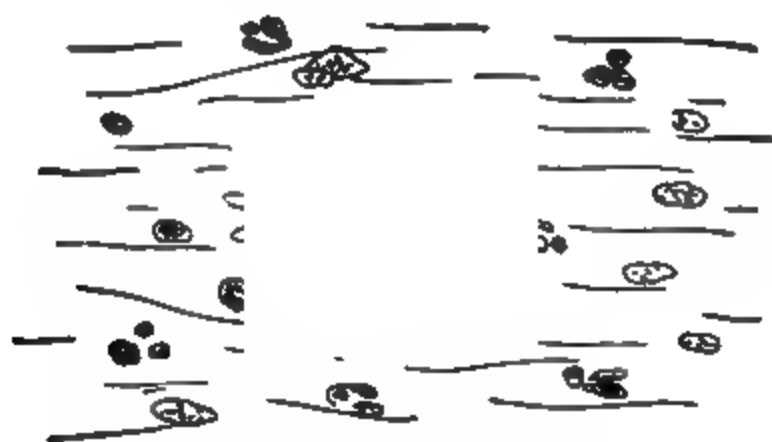
diseased tissue or with cultures results in the production of pathological lesions referable to the organism; again no effect follows upon such inoculation. As seen in the tissues by microscopic examination, actinomycetes may appear as long, convoluted, irregularly staining, beaded, branching threads, or as clumps of short, markedly beaded, sometimes branched rods. At times a clump of the short or longer threads is encountered in the tissues that gives the distinct impression of mycelial structure.

Some of the varieties that have been described are best demonstrated in the tissues or exudates by the Gram or Gram-Weigert method of staining; others are decolorized by this process, and are rendered visible only by the simpler procedures. Some of them are to a limited extent proof against the action of acid decolorizers. Though many accounts of the presence of these morphological types in a variety of conditions have been recorded, the descriptions in the main are meagre and often insufficient for identification. A few, however, have been found so constantly in association with more or less definite clinical and pathological conditions that a brief description of them may be of service.

Actinomyces Bovis (also commonly known as streptothrix actinomyces, actinomyces fungus, ray fungus) was first observed by von Langenbeck in a case of vertebral caries in 1845. According to Bollinger, the fungus had been seen by Hahn a number of years before in museum specimens, but had been regarded by him as a penicillium. The name actinomyces or ray fungus originated with Harz. It is constantly to be detected in the tissues and exudates of the disease of cattle known as actinomycosis, "lumpy jaw," "wooden tongue," etc. The typical tumor of this disease is characterized by inflammation, pus formation, excessive

new formation of connective tissue, abscesses, cavities, and sinuses. Viewed as a whole, the tumor presents points of resemblance to the osteo-sarcomatous, to the scrofulous or tuberculous, and to the cancerous processes. The disease occasionally occurs in man, and according to the point of entrance of the parasite may arise in the mouth, the pharynx, the lungs, the intestines, or the skin. In animals the disease is characterized by an excessive new formation of connective tissue, so that tumefaction is always a conspicuous peculiarity. In man, on the other hand, suppuration is the most prominent feature.

FIG. 79



Actinomyces fungus in pus. Fresh, unstained preparation. Magnified about 500 diameters.

If the purulent discharge from an actinomycotic tumor be examined fresh, it will be found to contain tiny yellow (sulphur color as a rule) clumps. If these be examined, unstained, in a drop of physiological salt solution or water under the microscope, they will be found to be made up of a rosette-like mass of closely interwoven threads. (See Fig. 77.) At the centre the mass may show the presence of spherical, coccus-like bodies or granules, while at the periphery the free ends of the threads are more or less distinctly

bulbous or nodular, or both, and they may show branching. Sometimes the free ends of the threads are only slightly or not at all swollen.

These mycelia—the actinomyces—may be stained by the ordinary aniline dyes, or by the Weigert or the Gram method, though by either of these procedures their full structure is not, as a rule, brought out. The reason for this is that the terminal bulbs are not due to enlargement of the thread itself, but rather to a colloid degeneration of its enveloping sheath. This colloid matter, having different microchemical reactions from the enclosed thread, requires different reagents to stain it. The entire structure may be seen when the fungus is stained first by the Gram method, and subsequently with eosin or saffranin. For the demonstration of the fungus in sections, the method of Mallory gives satisfaction. It is as follows; Stain the section on the slide with gentian-violet; clear and dehydrate with aniline oil in which a little basic fuchsin has been dissolved; remove the aniline oil-fuchsin with xylol, and mount in xylol balsam. In sections treated in this way the coccus-like central masses and the filamentous threads making up the mass of the mycelium are stained blue; the club-like extremities of the thread are red. Often the red-stained hyaline material is seen to be penetrated to its extremity by a sharply defined blue thread.

Cultivation of the fungus from the actinomycotic pus presents difficulties for the following reasons: Not all the mycelia seen by microscopic examination are living; as a rule they grow slowly even under the best of circumstances; and generally there are many other, more rapidly growing, living organisms in the pus. When pure cultures are obtained, it grows (according to Boström) on all the ordinary

artificial media. It develops at room-temperature, but better at that of the body.

It grows both with and without oxygen.

The young colonies appear as grayish points composed of a felt-work of fine threads. As the colonies become older they become denser and more opaque. Very old colonies are almost leathery in consistency. On blood-serum the growth after a time assumes a salmon, an orange, or a yellowish-red color. Growth on gelatin is accompanied by slow liquefaction.

A yellowish-red growth, limited in extent, occurs on potato. It causes no clouding of bouillon, but grows as cottony clumps that sink to the bottom.

The bulbous extremities seen upon the mycelial threads fresh from the pus are not usually seen under conditions of artificial cultivation. They are sometimes observed in colonies located in the depths of solid media. The white, powdery coating seen on old colonies represents the so-called "spores." They are not, however, resistant to heat, being destroyed, according to Domec, by 75° C. in five minutes.

Bovines are the animals most frequently affected. The disease has been seen in swine, dogs, and horses.

The most common seat of the disease is the jaw, and this, together with the fact that particles of fodder, such as bits of grain, chaff, straw, and barley beard, have been detected in the diseased tissues in association with the causative fungus, has led to the belief that the parasite gains access to the tissues with such foodstuffs. It has not, however, been recognized outside the animal body. The disease is apparently not transmissible from animal to animal or from animal to man. Inoculation of animals with pure cultures is usually negative, although nodular formations have fol-

lowed the injection of large quantities into the peritoneal cavity of rabbits. In Boström's cases the nodules presented only a few of the club-shaped extremities of the threads, and there was no evidence of multiplication of the fungus; while in the experiments of Israel and Wolf it is said there developed, in from four to seven weeks after intraperitoneal inoculation, larger and smaller tumors in which typical mycelia were present, and from which the fungus was obtained in pure culture.

Actinomyces Maduraë.—This organism is supposed to be concerned in the causation of mycetoma or Madura foot. Two varieties of mycetoma are known, viz., the pale or ochroid and the black or melanoid. Save for its occurrence in the foot, mycetoma is almost a counterpart of actinomycosis; and the suspicion of their identity is by no means lessened by the fact that the actinomyces constantly associated with the ochroid variety is to all intents and purposes identical with actinomyces bovis. It differs from that organism only in such minor details as to leave little doubt that they are very closely related, if not identical, so that a description of the one serves equally to aid in the identification of the other.

The investigations of Wright,¹ conducted upon a case encountered in Boston, point to another type of parasite as the causative factor in the black mycetoma. Instead of an actinomyces, Wright found a true mold. He expresses the opinion that the pale mycetoma is, etiologically, actinomycosis, and that the black is a hyphomycetic infection.

The fungus encountered and isolated in pure culture by

¹ A Case of Mycetoma (Madura Foot), Journal of Experimental Medicine, 1898, iii, 421.

Wright presented the following characteristics: As obtained from the affected tissues, the mycelia under the microscope appear as black or brown mulberry-like masses less than one millimeter in diameter. They are hard, rather brittle, and difficult to break up under the cover-glass. On soaking them in a strong solution of sodium hydroxide they become softened and the structure of the fungus-mass can be made out. Under high magnifying power these masses are found to consist of pigment-granules, ovoid translucent bodies, and distinctly branching separate hyphæ. Sometimes these latter exhibit dilatations or varicosities of their segments. The periphery of a fungous mass shows the presence of club-shaped hyphæ, closely set and radially arranged. From such masses growth on artificial culture-media may be obtained. When transferred direct from the tissues to artificial media, growth in every case starts from the granule about four or five days after it is placed upon the culture-media.

On solid media it first appears as delicate tufts of whitish filaments. These in the course of a few days increase in number and length, and, in the case of the potato, form a dense whitish or pale-brown felt-work having a tendency to spread widely.

In pure cultivation it is seen as long, branching hyphæ with delicate transverse septa. In old forms the hyphæ may be swollen at the points marked by the septa, and may then appear as strings of plump oval segments. The filaments have a definite wall, inclosing granules and pale areas. No spore-bearing organs are seen.

On potato, it grows as a dense, widely spreading, velvety membrane; pale brown at the centre and white at the periphery. The potato takes on a dark-brown color and

becomes very moist and dark; coffee-colored granules appear upon the surface of the growth.

In bouillon the growth assumes a puff-ball appearance. The medium assumes a deep coffee-brown color, and ultimately a mycelium growth appears upon the surface and throughout the fluid.

When grown in potato infusion (20 grams of potato boiled in water, filtered and made up to a liter), the growth is characterized by the appearance of black granules in the midst of the mycelium. The black granules consist of closely packed spherical or polyhedral cells, together with some short, thick segmented hyphæ. The walls of these cells have a black appearance, and masses of them are black and opaque under the microscope.

On agar-agar, growth appears as a grayish mesh-work of widely spreading filaments. In old cultures black granules (sclerotia) appear among the filaments. No growth occurs in the depth of the medium.

No results were obtained by the inoculation of animals with either the material direct from the tissues or with pure cultures.

The tissue from which this fungus was obtained consisted, briefly, of a more or less atypical connective-tissue new-growth, with numerous areas of suppuration marked by the presence of the black granules just described.

On histological study of the tumor the primary effect produced by the parasite appears to be the development of nodules of epithelial cells and of giant cells from the tissues immediately about them. Later, suppuration of the nodules and abscess formation occur. This in time gives rise to excessive development of granulation and connective tissue.

Actinomyces Farcinicus (bacille du farcin des bœufs (Nocard); oöspora farcinica; actinomyces bovis farcinicus).—This organism was discovered by Nocard (1888) in a disease of cattle that is suggestive of farcy as seen in horses. The lesions consist of chains of enlarged subcutaneous lymph-glands, which on examination are found to be in a condition somewhat simulating tuberculosis. Similar nodules are sometimes encountered in the internal organs.

By microscopic examination the organism is seen as long, branching threads consisting of short segments.

It is non-motile. Spore-formation is questionable, Nocard having seen it, while Lehmann and Neumann have not. The organism may be stained by the ordinary methods, and also by the Gram-Weigert process. It grows on all the ordinary culture-media, and at both room- and body-temperature, especially well at the latter. It is aërobic.

Colonies in agar-agar reach a size of from 1 to 2 mm.; are yellowish-white in color, irregular in outline, and have the appearance of a glazed, membranous mass.

On gelatin, the growth is much slower, so that after ten days the colonies appear as tiny translucent round glistening points. Under low power of the microscope these colonies are sharply circumscribed, grayish or greenish in color, and are without characteristic structure.

Growth in bouillon is characterized by a tough, slimy sediment, and at times by more or less of pellicle-formation. Pellicle-formation is encouraged by the addition of glycerin. The bouillon is not uniformly clouded by the growth.

In milk, it causes an alkaline reaction, solution of casein, but no coagulation.

On potato, it grows slowly as a dull yellowish-white dry membrane.

Bovines, sheep, and guinea-pigs are susceptible to inoculation; rabbits, dogs, cats, horses, and asses are not.

When pure cultures are injected into either the circulation or the peritoneal cavity of guinea-pigs, death ensues in from nine to twenty days. The autopsy reveals diffuse pseudotuberculosis of the omentum. Within the pseudotubercles the organism is seen as long, branching threads, often matted together as a true mycelium.

By subcutaneous inoculation only the neighboring lymph-glands are affected.

The disease farcin des bœufs is said to be more common in Guadeloupe than elsewhere.

Actinomyces Eppingeri.—This organism was discovered by Eppinger in an abscess of the brain. He regarded it as a cladothrix, and gave to it the designation cladothrix asteroides. It grows well in pure culture under artificial conditions, and is pathogenic for animals. In the case studied by Eppinger the organism was present not only in the abscess, but also in the meninges of the brain and cord and in the bronchial and supraclavicular lymph-glands. There is no doubt of its causal relation to the conditions.

In pure culture it grows well on ordinary media. It appears as long, branching threads, many of which are composed of short quadratic segments. Spores are not formed. Motility is doubtful; it has been observed by Eppinger, while Lehmann and Neumann failed to detect it. It stains both by the ordinary dyes and by the method of Gram. It grows scarcely, if at all, under anaërobic conditions. It grows at room-temperature, but much better at the temperature of the body. The best growth is observed on nutrient agar-agar containing 2 per cent. of glucose. The colonies on the surface of glucose-agar-agar appear as

yellowish-white, round, finely granular, dull patches that are surrounded by a narrow paler zone. In the depths of the medium they do not develop beyond very small points.

On gelatin the growth is very slow; there is no liquefaction, and after a time the colonies take on an orange-red color.

Bouillon is not uniformly clouded. Growth takes place on the surface in the form of a whitish pellicle, in which dense white masses may be seen. These latter increase in size, become detached, and fall to the bottom of the vessel, to collect as mycelium-like sediment.

On potato, growth begins as a coarsely granulated white layer, which becomes gradually red in color. It is ultimately covered by a fine, hair-like growth.

Both rabbits and guinea-pigs are susceptible to its pathogenic action. When injected into either the circulation, the peritoneal cavity, or beneath the skin, there develop in from one to four weeks a condition closely simulating tuberculosis ("pseudotuberculosis cladothrica"). The organism quickly loses its pathogenic properties under artificial cultivation.

Actinomyces Pseudotuberculosis.—In 1897 Flexner detected this organism in a consolidated and caseous lung. The condition suggested tuberculosis. The lesion consisted mainly of an inflammatory exudation that had undergone caseation, but in addition there were present isolated nodules that in size and general appearance were difficult to distinguish from miliary tubercles. Giant cells were not seen. The streptothrix was abundant in the lung, appearing as masses of convoluted, branching threads. The contours of the rods were not quite uniform, the staining was irregular, and occasionally a thread was seen that, toward its extrem-

ity, appeared to be breaking up into short segments. No coccus-like forms were seen. It is stained best by the Weigert method, when deeply stained masses separated from one another by more or less clear spaces are to be detected. The organism was not obtained in culture, and no effect was produced on guinea-pigs by subcutaneous inoculation with bits of the diseased lung.

CHAPTER XXII.

Glanders—Characteristics of the Disease—Histological Structure of the Glanders Nodule—Susceptibility of Different Animals to Glanders—The Bacterium of Glanders; Its Morphological and Cultural Peculiarities—Diagnosis of Glanders—Mallein.

THE disease is generally known as glanders when the mucous membrane of the nostrils is affected, and as farcy when the subcutaneous lymphatics are the principal sites of involvement.

Though most commonly seen in the horse and ass, glanders is not rarely met with in other animals, and is occasionally encountered in man. When occurring in the horse its primary seat is usually upon the mucous membrane of the nostrils. It appears in the form of small gray nodules, about which the membrane is congested and swollen. These nodules ultimately coalesce to form ulcers. There is a profuse slimy discharge from the nostrils during the course of the disease. The primary lesion may extend from its seat in the nose to the mouth, larynx, trachea, and ultimately to the lungs. Its secondary manifestations are observed along the lymphatics that communicate with the initial focus; in the lymphatic glands, and as metastatic foci in the internal organs.

Less frequently the disease is seen to begin beneath the skin, particularly in the region of the neck and breast. When in this locality the subcutaneous lymphatics become involved, and are converted into indurated, knotty cords—"farcy-buds"—easily discernible from without.

In man it usually occurs in individuals who have been in attendance upon animals affected with the disease. It may occur upon the mucous membrane of the nares; but its most frequent expressions are in the skin and muscles, where appear abscesses, phlegmons, erysipelas-like inflammations, and local necrosis closely resembling carbuncles. Metastases to the lungs, kidneys, and testicles, as in the horse, may also be seen.

When occurring upon the mucous membrane glanders is characterized by the presence of gray nodules, about as large as a pin-head, that closely resemble miliary tubercles in their naked-eye appearance. These consist histologically of granulation-tissue—*i. e.*, of small round cells, very similar to proliferating leukocytes—of some lymph-cells, and, in the earliest stages, of a small portion of necrotic tissue. As they grow older, and the process advances, there is a tendency to central necrosis, with the ultimate formation of a soft, yellow, creamy, pus-like material. Though strikingly like miliary tubercles in certain respects in the early stages, they present, nevertheless, decided points of difference when examined more in detail.

The round-cell infiltration of the glanders nodule consists essentially of polymorphonuclear leukocytes, while that of the miliary tubercle partakes more of the nature of a lymphocytic infiltration; in the later stages of the process the glanders nodule breaks down into a soft, creamy matter, very analogous to ordinary pus, while in the later stages of the miliary tubercle the tendency is to an amalgamation of its histological constituents, and ultimately to necrosis with caseation. The giant-cell formation common to tuberculosis is never seen in the glanders nodule. As Baumgarten aptly puts it: "The pathological manifestations of glanders,

from the histological aspect, stand midway between the *acute purulent* and the *chronic* inflammatory processes.”¹ Evidently these differences are only to be explained by differences in the nature of the causes that underlie the several affections. We have studied the characteristics of bacterium tuberculosis; we shall now take up the bacillus of glanders and note the striking differences between them.

BACTERIUM MALLEI (LÖFFLER), MIGULA, 1900.

SYNONYMS: *Bacillus mallei* (Löffler), 1886; *Rotz bacillus*, Kransfeld, 1887.

In 1882 Löffler and Schütz discovered in the diseased tissues of animals suffering from glanders a bacterium that, when isolated in pure culture and inoculated into susceptible animals, possesses the property of reproducing the disease with all its clinical and pathological manifestations. It is therefore the cause of the disease.

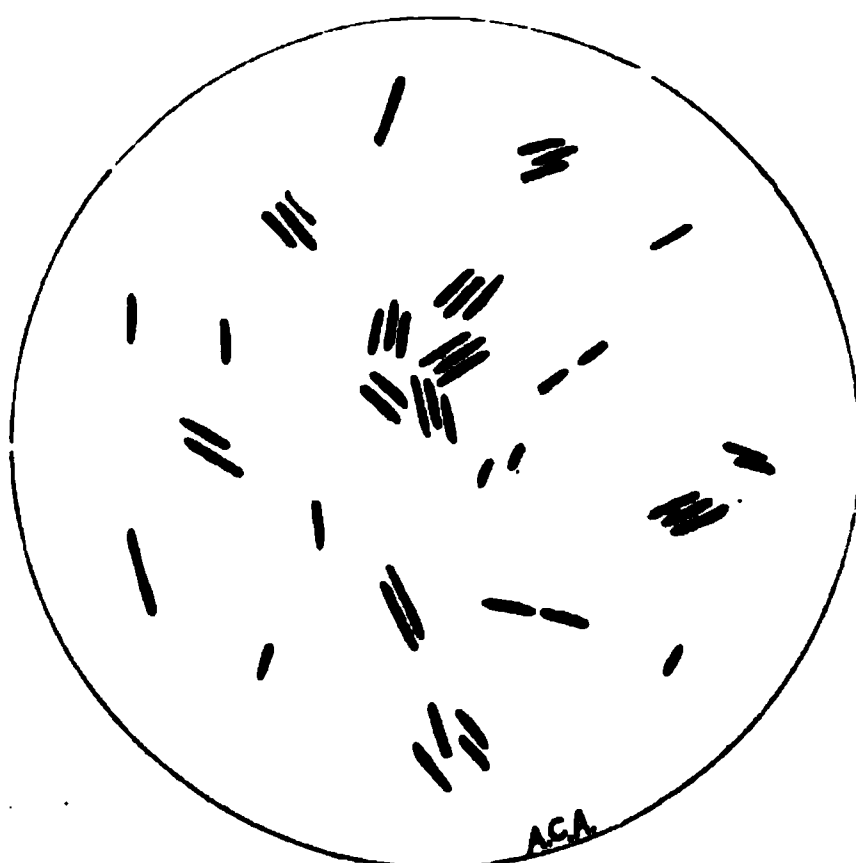
This organism is a rod, with rounded or slightly pointed ends. It usually stains somewhat irregularly. (See Fig. 78.) When examined in stained preparations its continuity is marked by alternating darkly and lightly stained areas. It is usually seen as a single rod, but may occur in pairs, and less frequently in longer filaments.

The question as to its spore-forming property is still an open one, though the weight of evidence is in opposition to the opinion that it possesses this peculiarity. Certain observers claim to have demonstrated spores in the bacteria by particular methods of staining; but this statement can

¹ For a further discussion of the pathology and pathogenesis of this disease, see *Lehrbuch der pathologischen Mykologie*, by Baumgarten, 1890. See, also, Wright, *The Histological Lesions of Acute Glanders in Man*, *Journal of Experimental Medicine*, i, 577.

have but little weight when compared with the behavior of the organism when subjected to more conclusive tests. For example, it does not, at any stage of development, resist exposure to 3 per cent. carbolic acid solution for longer than five minutes, nor to 1:5000 sublimate solution for more than two minutes. It is destroyed in ten minutes in some experiments, and in five in others, by a temperature of 55° C.;

FIG. 80



Bacterium mallei, from culture.

and when dried it loses its vitality, according to different observers, in from thirty to forty days; all of which speak directly against this being a spore-bearing bacillus.

It is not motile, and does not therefore possess flagella.

It grows readily on ordinary nutrient media at from 25° to 38° C.

Upon nutrient agar-agar, both with and without glycerin, it appears as a moist, opaque, glazed layer, with nothing

characteristic about it. This is true both for smear-cultures and for single colonies.

Its growth on gelatin is much less voluminous than on media that can be kept at higher temperature, though it does grow on this medium at room-temperature without causing liquefaction.

Its growth on blood-serum is in the form of a moist, opaque, slimy layer, inclining to a yellowish or dirty, brownish-yellow tinge. It does not liquefy the serum.

On potato its growth is moderately rapid, appearing in from twenty-four to thirty-six hours at 37° C. as a moist, amber-yellow, transparent deposit having somewhat the appearance of honey; this becomes deeper in color and denser in consistence as growth progresses, and finally takes on a reddish-brown color; at the same time the potato about it becomes darkened.

In bouillon it causes diffuse clouding, with ultimately the formation of a more or less tenacious or ropy sediment.

In milk to which a little litmus has been added it causes the blue color to become red or reddish in from four to five days, and quite red after two weeks at 37° C. At the same time the milk separates into clear whey and a firm clot of casein.

Its reactions to heat are very interesting. At 42° C. it will often grow for twenty days or more. It will not grow at 43° C., and if exposed to this temperature for forty-eight hours it is destroyed. It is killed in five hours when exposed to 50° C., and in five minutes by 55° C.

It grows both with and without oxygen; it is therefore *facultative* as regards its relation to this gas.

On cover-slips it stains readily with all the basic aniline dyes, and, as a rule, as stated, presents conspicuous irregu-

larities in the way that it takes up the dyes, being usually marked by deeply stained areas that alternate with points at which it either does not stain at all or only slightly.

The animals susceptible to infection by this organism are horses, asses, field-mice, guinea-pigs, and cats. Baumgarten records cases of infection in lions and tigers that were fed, in menageries, with flesh from horses affected with the disease. Rabbits are but slightly susceptible; dogs and sheep still less so. Man is susceptible, and infection not rarely terminates fatally. White mice, common gray house-mice, rats, cattle, and hogs are insusceptible.

Inoculation Experiments.—The most favorable animal upon which to study the pathogenic properties of this organism in the laboratory is the common field-mouse. When inoculated subcutaneously with a small portion of a pure culture of bacterium mallei death ensues in about seventy-two hours. The most conspicuous tissue-changes will be enlargement of the spleen, which is at the same time, almost constantly, studded with minute gray nodules, the typical glanders nodule. They are rarely present in the lungs, but may frequently be seen in the liver. From these nodules the glanders bacillus may be obtained in pure culture. With the exception of the characteristic nodules, the disease as seen in this animal presents none of the features that it displays in the horse and ass. The clinical and pathological manifestations resulting from inoculation of guinea-pigs are much more faithful reproductions. The animal lives usually from six to eight weeks after inoculation, and during this time becomes affected with a group of most interesting and peculiar pathological processes. The specific inflammatory condition of the mucous membrane of the nostrils is almost always present. The joints become swollen

and infiltrated to such an extent as often to interfere with the use of the legs. In male animals the testicles become enormously distended with pus, and on closer examination a true orchitis and epididymitis are seen to be present. The internal organs, particularly the lungs, kidneys, spleen, and liver, are usually the seat of the nodular formations characteristic of the disease. From all of these disease-foci the bacillus causing them can be isolated in pure culture.

Staining in Tissues.—Though always present in the diseased tissues, considerable trouble is usually experienced in demonstrating the bacteria by staining-methods. The difficulty is due to the fact that the bacteria are very easily decolorized, and in tissues stained by the ordinary processes are robbed of their color even by the alcohol with which the tissue is rinsed and dehydrated. If we will remember not to employ concentrated stains, and not to expose the sections to the stains for too long a time, but little treatment with decolorizing-agents is necessary, and very satisfactory preparations will be obtained. A number of methods have been suggested for staining the glanders bacilli in tissues, and if what has been said will be borne in mind, no difficulty should be experienced. Two satisfactory methods that we have used for this purpose, though perhaps no better than some of the others, are as follows:

a. Transfer the sections from alcohol to distilled water. This lessens the intensity with which the stain subsequently takes hold of the tissues, by diminishing the activity of the diffusion that would occur if they were placed from alcohol into watery solutions of the dyes. Transfer from distilled water to the slide, absorb all water with blotting-paper, and stain with two or three drops of

Carbol-fuchsin	10 c.c.
Distilled water	100 c.c.

for thirty minutes; absorb all superfluous stain with blotting-paper, and wash the section three times with 0.3 per cent. acetic acid, not allowing the acid to act for more than ten seconds each time. Remove all acid from the section by carefully washing in distilled water; absorb all water by gentle pressure with blotting-paper; and finally, at *very moderate heat*, or with a small bellows (Kühne), dry the section completely on the slide. When dried clear in xylol and mount in xylol balsam.

b. Transfer the sections from alcohol to distilled water; from water to the dilute fuchsin solution, and gently warm (about 50° C.) for fifteen to twenty minutes. Transfer sections from the staining-solution to the slide, absorb all superfluous stain with blotting-paper, and then treat them with 1 per cent. acetic acid from one-half to three-quarters of a minute. Remove all trace of acid with distilled water, absorb all water by gentle pressure with blotting-paper, and then treat the sections with absolute alcohol by allowing it to flow over them drop by drop. For small sections three or four drops are sufficient. Under no circumstances should the alcohol be allowed to act for more than one-quarter of a minute. Clear in xylol and mount in xylol balsam.

By method *b* the tissues are better preserved than by method *a*, by which they are dried.

In properly stained tissues the bacteria will be found most numerous in the centre of the nodules, becoming fewer as we approach the periphery. They usually lie between the cells, but at times may be seen almost filling some of the epithelial cells, of which the nodule contains more or less. They are always present in these nodules in the tissues; they are rarely present in the blood, and, if so, in only small numbers.

Diagnosis of the Disease by the Agglutination Test.—The quickest and surest method of recognizing the disease is by the specific agglutinating effect of the serum of the diseased animal upon the organism of the disease. Many different plans have been recommended. That of Moore, of Cornell University, is one of the most trustworthy. He recommends a test emulsion made by suspending a glycerin-agar culture of glanders bacilli in physiological salt solution. This is then exposed to 60° C. for two hours, whereby the bacteria are killed, and is finally preserved by the addition of 0.5 per cent. carbolic acid. To this suspension the serum of the suspected animal is added in varying proportions until a distinct clumping and sedimentation of the bacteria is observed. Whenever done in a small test-tube of about 0.5 cm. diameter this reaction manifests itself as a gradual clarification of the milky fluid and the accumulation of a mass on the bottom of the tube. Normal horse serum in a dilution of 1 to 300 to 1 to 200 causes the agglutination, while that from glanders animals does the same in from 1 to 3200 to 1 to 500 dilution. The “complement fixation” reaction may also be applied both for the recognition of the condition—*i. e.*, for detecting the specific antibodies in the tissues or fluids, as well as for the identification of the specific exciter of the condition—*i. e.*, the antigen. (See that reaction.)

Mallein.—The sterile filtered products of growth of the glanders bacillus in fluid media represent what is known as *mallein*—a solution of compounds that bear to glanders a relation analogous to that which tuberculin bears to tuberculosis. It is used with considerable success as a diagnostic aid in detecting the existence or absence of deep-seated manifestations of the disease, the glanderous animal reacting

(manifested by elevations of body-temperature greater than 1.5° C.) to subcutaneous injections of mallein in from four to ten hours, while an animal not so affected gives no such reactions.

Mallein is prepared from old glycerin-bouillon cultures of the glanders bacterium by steaming them for several hours in the sterilizer, after which they are filtered through unglazed porcelain.

By some it is said that the repeated injection of mallein in small doses confers immunity from infection by bacterium mallei upon animals so treated; an opinion that is entirely in accord with the principles underlying the artificial induction of immunity in general.

CHAPTER XXIII.

Bacterium (Syn. Bacillus) Diphtheriæ—Its Isolation and Cultivation—Morphological and Cultural Peculiarities—Pathogenic Properties—Variations in Virulence—Bacterium Pseudodiphtheriticum—Bacterium Xerosis—Diphtheria Antitoxin.

FROM the gray-white deposit on the fauces of a diphtheritic patient prepare a series of cultures in the following way:

Have at hand five or six tubes of Löffler's blood-serum mixture. (See chapter on Media.) Pass a stout platinum needle, which has been sterilized, into the membrane and twist it around once or twice, or brush it gently over the surface of the membrane. Without touching it against anything else rub it carefully over the surface of one of the serum-tubes; without sterilizing it pass it over the surface of the second, then the third, fourth, and fifth tubes. Place these tubes in the incubator. Then prepare cover-slips from scrapings from the membrane on the fauces. If the case is one of true diphtheria, the tubes will be ready for examination on the following day.

The reason that plates are not made in the regular way in this examination is that the bacillus of diphtheria develops much more luxuriantly on the serum mixture, from which plates cannot be made, than it does on the media from which they can be made. The method employed, however, insures a dilution in the number of organisms present, and this, in addition to the fact that the blood-serum mixture is a much more favorable medium for the rapid development of the diphtheria organism than of the other organisms present,

makes its isolation by this method a matter of but little difficulty.

After twenty-four hours in the incubator the tubes present a characteristic appearance. Their surfaces are marked by more or less irregular patches of a white or cream-colored growth, which is usually more dense at the centre than at the periphery. Except now and then, when a few orange-colored colonies may be seen, these large irregular patches are more conspicuous objects on the surface of the serum. Occasionally, almost nothing else appears.

The cover-slips made from the membrane at the time the cultures were prepared will be found on microscopic examination to present, in many cases, a great variety of organisms; but conspicuous among them will be noticed slightly curved bacilli of irregular size and outline. In some cases they will be more or less clubbed at one or both ends; sometimes they appear spindle in shape, again as curved wedges; now and then they are irregularly segmented. They are rarely or never regular in outline. If the preparation has been stained with Löffler's alkaline methylene-blue solution, many of these irregular rods are seen to be marked by circumscribed points in their protoplasm which stain very intensely—they appear almost black. This irregularity in outline is the morphological characteristic of *bacillus diphtheriæ* of Löffler.

It must be remembered, however, that the diagnosis of diphtheria should not under all circumstances be made from the examination of cover-slip preparations alone, especially when they are stained only by the usual method—*i. e.*, with Löffler's methylene-blue. There are other organisms present in the mouth-cavity, particularly in the mouths of persons having decayed teeth, the morphology of which

is so like that of the bacillus of diphtheria that they might easily be mistaken for that organism if subjected to only the usual method of microscopic examination; and again, the genuine diphtheria organism is sometimes found in the mouth-cavities of healthy persons in attendance upon diphtheria cases, such persons being at the time insusceptible to the pathogenic activities of the organism. In the vast majority of instances, however, where the clinical condition of the patient justifies a suspicion of diphtheria, a microscopic examination alone of the deposit in the throat, made by an experienced person, will serve to confirm or contradict this opinion, and such examinations very frequently reveal the diphtheritic nature, etiologically speaking, of mild conditions of the throat which are not associated with grave constitutional manifestations.

BACTERIUM DIPHTHERIÆ (LÖFFLER), MIGULA, 1900.

SYNONYMS: *Bacillus diphtheriæ*, Löffler, 1884; *Klebs-Löffler bacillus*; *Corynebacterium diphtheriæ*, Lehmann and Neumann, 1896.

Bacterium diphtheriæ, discovered microscopically by Klebs, and isolated in pure culture and proved to stand in causal relation to diphtheria by Löffler, can readily be identified by its cultural peculiarities and by its pathogenic activity when introduced into tissues of susceptible animals. In guinea-pigs and kittens the results of its growth are histologically identical with those found in the bodies of human beings who have died of diphtheria.

When studied in pure culture its morphological and cultural peculiarities are as follows:

MORPHOLOGY.—As obtained directly from the diphtheritic deposit in the throat of an individual sick of the disease,

it is sometimes comparatively regular in shape, appearing as straight or slightly curved rods with more or less pointed ends. More frequently, however, spindle- and club-shapes occur, and not rarely many of these rods stain irregularly; in some of them very deeply stained round or oval points can be detected.

When *cultures* are examined microscopically it is especially characteristic to find irregular, bizarre forms, such as rods with one or both ends swollen, and very frequently rods broken at irregular intervals into short, sharply defined segments, either round, oval, or with straight sides. Some forms stain uniformly, others in various irregular ways, the most common being the appearance of deeply stained granules in a lightly stained bacillus.

By a series of studies upon this organism when cultivated under artificial conditions we have found that its form and size depend very largely upon the nature of its environment. That is to say, its morphology is always more regular, and it is smaller on glycerin-agar-agar than on other media used for its cultivation; while upon Löffler's blood-serum the other extremes of development appear: here one sees, instead of the very short, spindle-, lancet-, club-shaped, always segmented and regularly staining forms as seen upon glycerin-agar-agar, long, sometimes extremely slender, sometimes thicker, irregularly staining threads that may be either clubbed or pointed at their extremities. They are, as a rule, marked by areas that stain more intensely than does the rest of the rod, and at times they may be a little swollen at the centre. These differences are so conspicuous that microscopic preparations from cultures from the same source, but cultivated in the one case on glycerin-agar-agar and in the other upon blood-serum, when placed side by side would

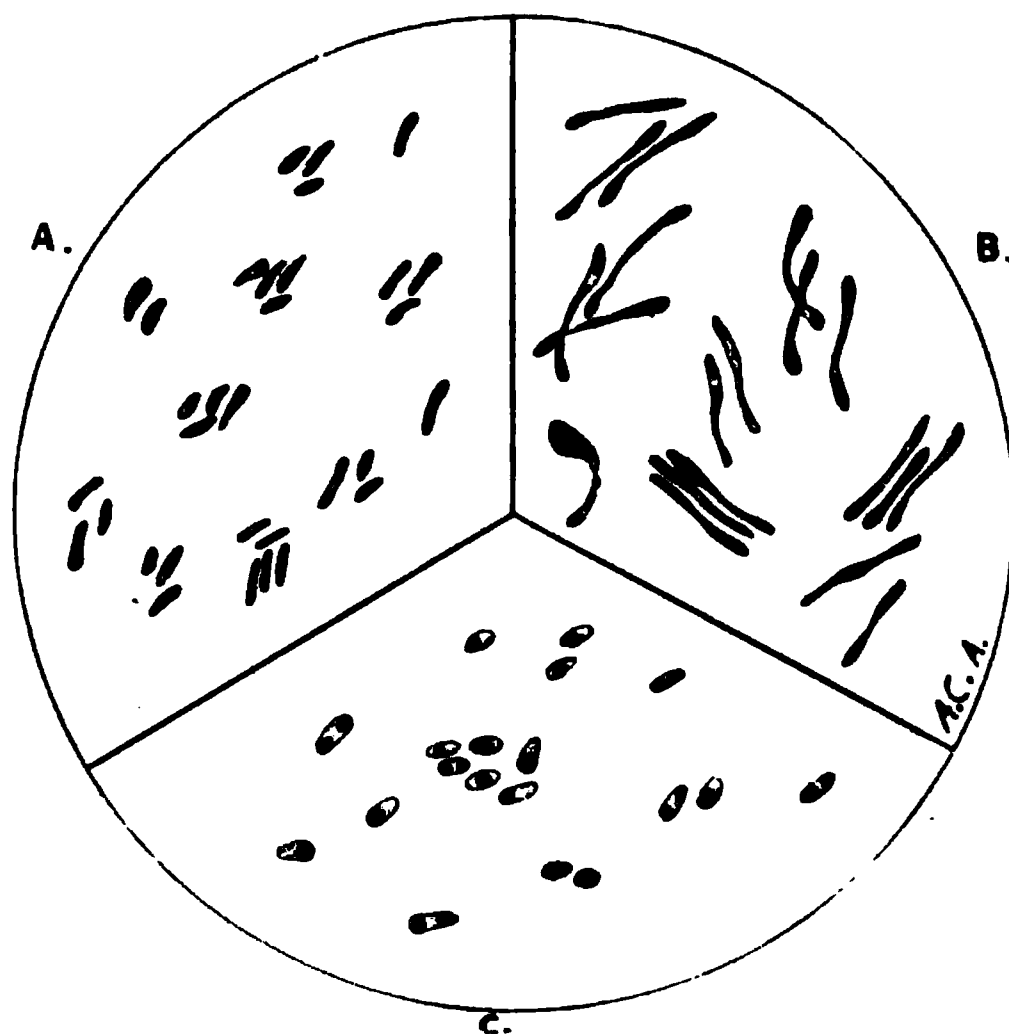
hardly be recognized as of the same organism, unless its peculiar behavior under these circumstances was already known. Another peculiar variation is that observed upon very slightly acid blood-serum. Here the rods appear swollen, and are usually contracted to oval or short, oblong bodies, which stain very faintly, and in which are usually located one or two very deeply staining round or oval points. Various authors have called attention to branching forms of this organism that are occasionally encountered, especially when cultivated upon albumin. We have never seen the branching diphtheria organisms under conditions that might reasonably be regarded as favorable to normal development; and in many thousand blood-serum cultures from cases of diphtheria that have been examined by competent bacteriologists at the laboratory of the Bureau of Health of Philadelphia, the branching forms of this organism have not been observed in a single instance. It is fair to assume, therefore, that this peculiar morphological variation of *bacillus diphtheriæ* is, under normal conditions of growth, comparatively rare.

On the other hand, if the organism be grown on media favorable to involution, such, for instance, as hard-boiled egg, or coagulated egg of slightly acid reaction, branching may be seen, but with it degenerated organisms are so conspicuous as to leave no doubt that the so-called branching and involution are attributable to the same cause, namely, unsuitable conditions of cultivation.

On plain nutrient agar-agar (that is, nutrient agar-agar without glycerin); on a medium consisting of dried commercial albumin dissolved in bouillon (about 10 grams of albumin to 100 c.c. of bouillon containing 1 per cent. of grape-sugar); in bouillon without glycerin, and in bouillon

to which a bit of hard-boiled egg has been added, the morphology of the organism is about intermediate, in both size and outline, between the forms seen upon glycerin-agar-agar and upon Löffler's blood-serum. There will appear about an equal number of short segmented and longer, irregularly staining forms; but in general the longest

FIG. 81



Bacterium diphtheriæ. A, its morphology on glycerin-agar-agar; B, its morphology on Löffler's blood-serum; C, its morphology on acid blood-serum mixture.

are rarely as long as the long forms seen on blood-serum, and throughout they are not so conspicuous for the irregularity of their staining.

In cultures made upon two sets of nutrient agar-agar tubes, differing only in the fact that one set contains glycerin to the extent of 6 per cent., while the other set contains

none, a noticeable difference in morphology can usually be made out: while the forms on the glycerin-agar-agar cultures are throughout small, and pretty regular in size, shape, and staining, those on the plain agar-agar are larger, stain less uniformly, vary more in shape, and when stained by Löffler's blue are not so regularly marked by pale transverse lines that give to them the appearance of being made up of numerous short segments.

Though the outline of this organism is more regular under some circumstances than others, it is nevertheless always conspicuous for its manifold variations in shape.

Growth on Serum Mixture.—The medium upon which bacillus diphtheriæ grows most rapidly and luxuriantly and which is best adapted for determining its presence in diphtheritic exudates, is, as has been stated, the blood-serum mixture of Löffler. (See chapter on Media.) On the blood-serum mixture the colonies of bacillus diphtheriæ grow so much more rapidly than the other organisms usually present in secretions and exudations in the throat that at the end of twenty-four hours they are often the only colonies that attract attention; and if others of similar size are present, they are generally of quite a different aspect. Its colonies are large, round, elevated, grayish-white or yellowish, with a centre more opaque than the slightly irregular periphery. The surface of the colony is at first moist, but after a day or two becomes rather dry in appearance.

A blood-serum tube studded with coalescent or scattered colonies of this organism is so characteristic that one familiar with the appearance can anticipate with tolerable certainty the results of microscopic examination.

Glycerin-agar-agar.—Upon nutrient glycerin-agar-agar the colonies likewise present an appearance that readily may

be recognized. They are in every way more delicate in structure than when on the serum mixture. They appear at first, when on the surface, as very flat, almost transparent, dry, non-glistening, round points which are not elevated above the surface upon which they are growing. When slightly magnified they are seen to be granular, and to present an irregular central marking, which is denser and darker by transmitted light than the thin, delicate zone which surrounds it. As the colony increases in size the thin granular peripheral zone becomes broader, is usually marked

FIG. 82



Colonies of bacterium diphtheriæ on glycerin-agar-agar. *a*, colonies located in the depths of the medium; *b*, colonies just breaking out upon the surface of the medium; *c*, fully developed surface-colony.

by ridges or cracks, and its periphery is notched or scalloped. (Fig. 80, *c*.) These colonies are always quite dry in appearance. When deep down in the agar-agar they are coarsely granular. (Fig. 80, *a*.) They rarely exceed 3 mm. in diameter.

Gelatin.—On gelatin the colonies develop much more slowly than on media that can be retained at a higher temperature. They rarely present their characteristic appearances on gelatin in less than seventy-two hours. They then appear as flat, dry, translucent points, usually round in outline.

When magnified slightly the centre is seen to be more dense than the surrounding zone or zones, for they are sometimes marked by a concentric arrangement of zones. The periphery is irregularly notched. Like the colonies seen on agar-agar, they are granular, but are much more granular when seen in the depths of the gelatin than when on its surface. On gelatin the colonies rarely become very large; usually they do not exceed 1.5 mm. in diameter.

Bouillon.—In bouillon it usually grows in fine clumps, which fall to the bottom of the tube, or become deposited on its sides without causing diffuse clouding of the bouillon. Sometimes there are exceptions to this naked-eye appearance; the bouillon may be diffusely clouded; but if one inspect it very closely, particularly if he examine it microscopically as a hanging drop, the arrangement in clumps will always be detected, but the clumps are so small as not to be discernible by the unaided eye.

In bouillon kept at a temperature of 35°–37° C. a soft, whitish pellicle often forms upon the surface.

The reaction of the bouillon frequently becomes at first acid, and subsequently again alkaline, changes which can be observed in cultivations in bouillon to which a little rosolic acid has been added. This play of reactions has been attributed to the primary fermentation of the muscle-sugar often present in the bouillon. It does not occur when the medium is free from carbohydrates.

Potato.—On potato at a temperature of 35°–37° C. its growth after several days is invisible, only a thin, dry glaze appearing at the point at which the potato was inoculated. Microscopic examination of scrapings from the potato, after twenty-four hours at 35°–37° C., reveals a decided increase in the number of individual organisms planted.

Stab- and Slant-cultures.—In stab- and slant-cultures on both gelatin- and glycerin-agar-agar the surface-growth is seen to predominate over that along the track of the needle in the depths of the media.

Isolated colonies on the surface of either of the media in this method of cultivation present the same characteristics that have been given for the colonies on plates.

The growth in simple stab-cultures does not extend laterally very far beyond the point at which the needle entered the medium.

It is a non-motile organism.

It does not form spores.

It is killed in ten minutes by a temperature of 58° C.

It grows at temperatures ranging from 22° to 37° C., but most luxuriantly at the latter temperature.

Its growth in the presence of oxygen is more active than when this gas is excluded.

STAINING.—In cover-slip preparations made either from the fauces of a diphtheritic patient or from a pure culture of the organism it is seen to stain readily with the ordinary aniline dyes. It stains also by the method of Gram, but the best results are obtained by the use of Löffler's alkaline methylene-blue solution; this brings out the dark points in the protoplasmic body of the bacilli and thus aids in their identification.

For the purpose of demonstrating the Löffler bacillus in sections of diphtheritic membrane, both the Gram method and the fibrin method of Weigert give excellent results.

Pathogenic Properties.—When inoculated subcutaneously into the bodies of susceptible animals the result is not the production of septicemia, as is seen to follow the introduction into animals of certain other organisms with which

we shall have to deal, but the bacillus of diphtheria remains localized at the point of inoculation, rarely disseminating further than the nearest lymphatic glands. It develops at the point in the tissues at which it is deposited, and during its development gives rise to changes in the tissues which result entirely from the absorption of poisonous albumins generated by the bacteria in the course of their development.

In a certain number of cases¹ diphtheria bacilli have been found in the blood and internal organs of individuals dead of the disease; but all that has been learned from careful study of the secondary manifestations of diphtheria tends to the opinion that they are in no way dependent upon the immediate presence of bacteria, and that the occasional appearance of diphtheria bacteria in the internal organs is in all probability accidental, and usually unimportant.

By special methods of inoculation² (the injection of fluid cultures into the testicles of guinea-pigs) diphtheria bacilli *can be caused* to appear in the omentum; but this is purely an artificial manifestation of the disease, and one that is probably never encountered in the natural course of events. More rarely similar results follow upon subcutaneous inoculation.

If a very minute portion of either a solid or fluid pure culture of this organism be introduced into the subcutaneous tissues of a guinea-pig or kitten, death of the animal ensues in from twenty-four hours to five days. The usual changes

¹ Frosch, Die Verbreitung des Diphtherie-bacillus in Körper des Menschen, Zeit. für Hygiene und Infektionskrankheiten, 1893, Bd. xii, pp. 49-52; Booker, Archives of Pediatrics, August, 1893; Wright and Stokes, Boston Med. and Surg. Journ., March and April, 1895.

² Abbott and Ghiskey, A Contribution to the Pathology of Experimental Diphtheria, The Johns Hopkins Hospital Bulletin, No. 30, April, 1893.

are an extensive local edema, with more or less hyperemia and ecchymoses at the site of inoculation; swollen and red-dened lymphatic glands; increased serous fluid in the peritoneum, pleura, and pericardium; enlarged and hemorrhagic adrenal bodies; occasionally slightly swollen spleen; and sometimes fatty degeneration in the liver, kidney, and myocardium. In guinea-pigs, especially, the liver often shows numerous macroscopic dots and lines on the surface and penetrating the substance of the organ. They vary in size from a pin-point to a pin-head, and may be even larger. They are white and do not project above the surface of the capsule.

The bacteria are always to be found at the site of inoculation, most abundant in the grayish-white, fibrino-purulent exudate. They become fewer at a distance from this, so that the more remote parts of the edematous tissues do not contain them. They are found not only free, but contained in large number in leukocytes, some of which have fragmented nuclei, or have lost their nuclei. The bacteria within leukocytes, as well as some outside, frequently stain very faintly and irregularly, and may appear disintegrated and dead.

Culture-tubes inoculated from the blood, spleen, liver, kidneys, adrenal bodies, distant lymphatic glands, and serous transudates, generally yield negative results; and negative results are also obtained when these organs are examined microscopically for the bacteria.

Microscopic examination of the tissues at the site of inoculation, as well as of the liver, spleen, kidneys, lymphatic glands, and elsewhere, reveals the presence of localized foci of cell-death, characterized by a peculiar fragmentation of the nuclei of the cells of these parts.

This destruction of nuclei results in the formation of groups of irregularly shaped, deeply staining bodies, having at times the appearance of particles of dust, while again they may be much larger. Some of them are tolerably regular in outline, while others are irregularly crescentic, dumb-bell, flask-shape, whetstone-shape, or bladder-like in form. Occasionally nuclei having the appearance of being pinched or drawn out can be seen. At some points the fragments are grouped in isolated masses, indicating the location of the nucleus from the destruction of which they originated. These particles always stain much more intensely than do the normal nuclei of the part.¹ Oertel showed long before bacillus diphtheriæ was discovered that these peculiar alterations in cell nuclei, both in distribution and appearance, are characteristic of human diphtheria, and the demonstration of similar changes in animals inoculated with this organism is important additional proof that diphtheria is caused by it.

By the inoculation of certain animals an affection may be produced in all respects identical with diphtheria as it exists in man. If one open the trachea of a kitten and rub upon the mucous membrane a small portion of a pure culture of this organism, the death of the animal usually ensues in from two to four days. At autopsy the wound will be found covered with a grayish, adherent, necrotic, distinctly diphtheritic layer. Around the wound the subcutaneous tissues will be edematous. The lymphatic glands at the angle of the jaws will be swollen and reddened. The mucous membrane of the trachea at the point upon which the bac-

¹ See *The Histological Changes in Experimental Diphtheria*, also *The Histological Lesions Produced by the Toxalbumin of Diphtheria*, by Welch and Flexner, *Johns Hopkins Hospital Bulletin*, August, 1891, and March, 1892.

teria were deposited will be covered with a tolerably firm, grayish-white, loosely attached pseudo-membrane in all respects identical with the croupous membrane observed in the same situation in cases of human diphtheria. In the pseudo-membrane and in the edematous fluid about the skin-wound bacillus diphtheriæ may be found both in cover-slips and in cultures.

From what we have seen—the localization of the bacilli at the point of inoculation, their absence from the internal organs, and the changes brought about in the cellular elements of the internal organs—there is but one interpretation for this process, viz., that it is due to the production of a soluble poison by the bacteria confined to the site of inoculation, which, gaining access to the circulation, produces the changes that we observe in the tissues of the internal viscera.

This poison has been isolated from cultures of bacillus diphtheriæ, and is found to belong, not to the crystallizable ptomains, but to the toxins—bodies which, in their chemical composition, are analogous to the poison of certain venomous serpents. By the introduction of this toxin into the tissues of guinea-pigs and rabbits the same pathological alterations may be produced that we have seen to follow inoculation with the bacilli themselves, except, perhaps, the production of false membranes.

Under certain circumstances with which we are not acquainted bacillus diphtheriæ becomes diminished in virulence or may lose it entirely, so that it is no longer capable of producing death of susceptible animals, and may cause only a transient local reaction from which the animal entirely recovers. Sometimes this reaction is so slight as to be overlooked, and again careful search may fail to reveal

evidence of any reaction at all. These exhibitions of the extremes of its pathogenic properties, viz., death of the animal, on the one hand, and only very slight local effects on the other, was at one time thought to indicate the existence of two separate and distinct organisms that were alike in cultural and morphological peculiarities, but which differed in their disease-producing power. Further studies on this point have, however, shown that genuine bacillus diphtheriæ may possess almost all grades of virulence, and that absence of or diminution in virulence can hardly serve to distinguish as separate species those varieties that are otherwise alike; moreover, the histological conditions found at the site of inoculation in animals that have not succumbed, but in which only the local reaction has appeared, are in most cases characterized by the same changes that are seen at autopsy in animals in which inoculation has proved fatal.

In the course of their observations upon a large number of cases Roux and Yersin found that it was not difficult to detect, in the diphtheritic deposits of the same individual, bacteria of identical cultural and morphological peculiarities, but of very different degrees of virulence, and that with the progress of the disease toward recovery the less virulent varieties often became quite frequent.¹

There is, moreover, a mild form of diphtheria, etiologically speaking, affecting only the mucous membrane of the nares, known as membranous rhinitis, from which it is very common to obtain cultures in all respects identical with those from typical diphtheria, save for their inability to *kill* sus-

¹ It must not be assumed from this that the bacteria lose their virulence entirely, or that they *all* become attenuated with the establishment of convalescence, for this is contrary to what experience has shown to be the case.

ceptible animals. On inoculation these cultures produce only local reactions, but these are characterized histologically by the same kind of tissue-changes that follow inoculation with the fully virulent organism.

Clinically, membranous rhinitis is never such an alarming disease as is laryngeal or pharyngeal diphtheria, and, as stated, the organisms causing it are often of a low degree of virulence, though they are, nevertheless, genuine diphtheria bacteria.

For those organisms that are in all respects identical with the virulent bacillus diphtheriæ, save for their inability to kill guinea-pigs, the designation "pseudodiphtheritic bacillus" is usually employed; but from such observations as those just cited we are inclined to the opinion that *pseudodiphtheritic*, as applied to an organism in all respects identical with the genuine bacterium, except that it is not *fatal* to susceptible animals, is a misnomer, and that it would be more nearly correct to designate this organism as the attenuated or non-virulent diphtheritic bacterium, reserving the term "pseudodiphtheritic" for that organism or group of organisms (for there are probably several) that is enough like the diphtheria bacterium to attract attention, but is distinguishable from it by certain morphological and cultural peculiarities aside from the question of virulence.

It is a well-known fact that many pathogenic organisms—conspicuous among these being bacterium pneumoniæ, micrococcus aureus, streptococcus pyogenes, and the group of so-called "hemorrhagic septicemia" organisms—undergo marked variations in their pathogenic properties; and yet these organisms, when found either devoid of this peculiarity, or possessing it in a diminished degree, are not designated as "pseudo" forms, but simply as the organisms themselves,

the virulence of which, from various causes, has been modified.

It must nevertheless be admitted that in the course of microscopic examination of materials from various sources, including the pharynx, one occasionally encounters microorganisms whose morphology is so like that of the genuine bacterium diphtheriæ as to create suspicion, and yet they are at the same time sufficiently unlike it to make one cautious in forming an opinion as to their real nature.

Bacterium Pseudodiphtheriticum.—For a long time bacterium pseudodiphtheriticum was looked upon as being entirely harmless, and the only particular in which it was regarded as being of importance was in the fact that it was likely to be mistaken for bacterium diphtheriæ. The wide dissemination of this class of organisms and the demonstration of pathogenic effects in isolated instances has led to the more systematic study of members of this group of organisms.

Bacterium pseudodiphtheriticum, as found under different conditions, varies markedly in its morphologic and biologic characters. Some of the varieties have definite chromogenic properties, producing various shades of yellow- and orange-colored pigment, while others grow with a pink color.

The occurrence of bacterium pseudodiphtheriticum in pure culture in superficial abrasions showing a slight tendency to suppuration; the fact that these organisms, when injected into the peritoneal cavity of guinea-pigs, produce purulent peritonitis; that such organisms are frequently encountered in vaccine virus and in the pus of vaccination wounds; and that frequently in cases of mastitis in cows such organisms occur in large numbers in pure culture has led to the supposition that this group of organisms was

probably responsible for suppurations occurring under certain special conditions. With these facts in mind specimens of pus were derived from thirty cases with suppurating wounds in the University of Pennsylvania Hospital, and careful bacteriological examination of these specimens showed the presence of bacterium pseudodiphtheriticum in 43 per cent. of the cases. These organisms were always found in conjunction with one or more of the group of pyogenic organisms, and it is impossible to state how much of the effect was due to any one of the organisms present. It seems probable, however, in the light of what has been said, that these bacteria were present not merely as accidental invaders, but that in some way they contributed toward the results.

The fact that some of the organisms isolated from the pus, when inoculated into the peritoneal cavity of guinea-pigs, show distinct pyogenic properties gives strong support to the opinion that this group is of greater importance than was heretofore supposed. Repeated passage through guinea-pigs serves to so increase the pathogenic properties of these organisms that they cause the death of the animal in less than twenty-four hours with marked inflammatory reaction affecting the peritoneum as well as the abdominal organs.

The morphologic and biologic characters of some members of the group of bacterium pseudodiphtheriticum are suggestive of those of bacterium diphtheriæ. Other members of the group, however, are readily differentiated from bacterium diphtheriæ by either the morphologic or the biologic characters, or by both. Many of the members of the group produce very little acid when grown in carbohydrate media, and the slight degree of acidity produced is frequently

obliterated by a marked degree of subsequent alkali production. This fact is of special value in the differentiation from bacterium diphtheriæ.

**BACTERIUM XEROSIS (NEISSER AND KUSCHBERT),
MIGULA, 1900.**

SYNONYM: *Bacillus xerosis*, Neisser and Kuschbert, 1883.

Another organism which is also related in its morphologic and biologic characters to bacterium diphtheriæ is bacterium xerosis, first encountered by Kuschbert and Neisser in xerosis of the conjunctiva, and which has since been found on the conjunctiva by a number of investigators, in various diseases as well as in health.

The xerosis bacteria are less likely to be mistaken for bacterium diphtheriæ because they are somewhat smaller and have less tendency to show multiple striations. Usually they stain deeply at the poles with only one clear unstained band in the centre. It is only occasionally that a few striated organisms are encountered in a culture.

Biologically bacterium xerosis is readily differentiated from bacterium diphtheriæ because of the scant growth that takes place on the ordinary culture-media. On agar-agar the growth appears as small transparent colonies which have little tendency to coalesce. On gelatin the growth is slow, and frequently shows as minute, isolated colonies along the needle track. In litmus-milk a slight degree of acidity is produced. In bouillon the growth is so slight as to leave the medium practically unaltered. The growth on potato is slight and invisible.

Differentiation of Members of the Group.—Knapp¹ claims that a positive differentiation of the organisms may be made by merely inoculating the Hiss media containing dextrin and saccharose. If the dextrin is alone fermented, the organism is bacterium diphtheriæ, if only the saccharose is fermented, the organism is bacterium xerosis, and if neither of these carbohydrates is fermented, the organism is bacterium pseudodiphtheriticum.

Through the suggestion of Neisser² we are assisted in differentiating between bacillus diphtheriæ and the confusing forms. He has found that by the use of a particular staining method the appearance of bacterium diphtheriæ is characteristic. His differential method comprehends the following manipulations: the culture to be tested should be grown upon Löffler's blood-serum mixture solidified at 100° C.; it should develop at a temperature not lower than 34° C. and not higher than 36° C.; and it should not be younger than nine and not older than twenty-four hours. A cover-glass preparation made from such a culture is stained as follows:

(a) It is subjected to the following mixture for from one to three seconds:

Methylene-blue (Grübler's)	1 gram
Alcohol (96 per cent.)	20 c.c.

When dissolved, mix with

Acetic acid	50 c.c.
Distilled water	950 c.c.

(b) After thoroughly rinsing in water, it is stained for from three to five seconds in vesuvin (Bismarck-brown),

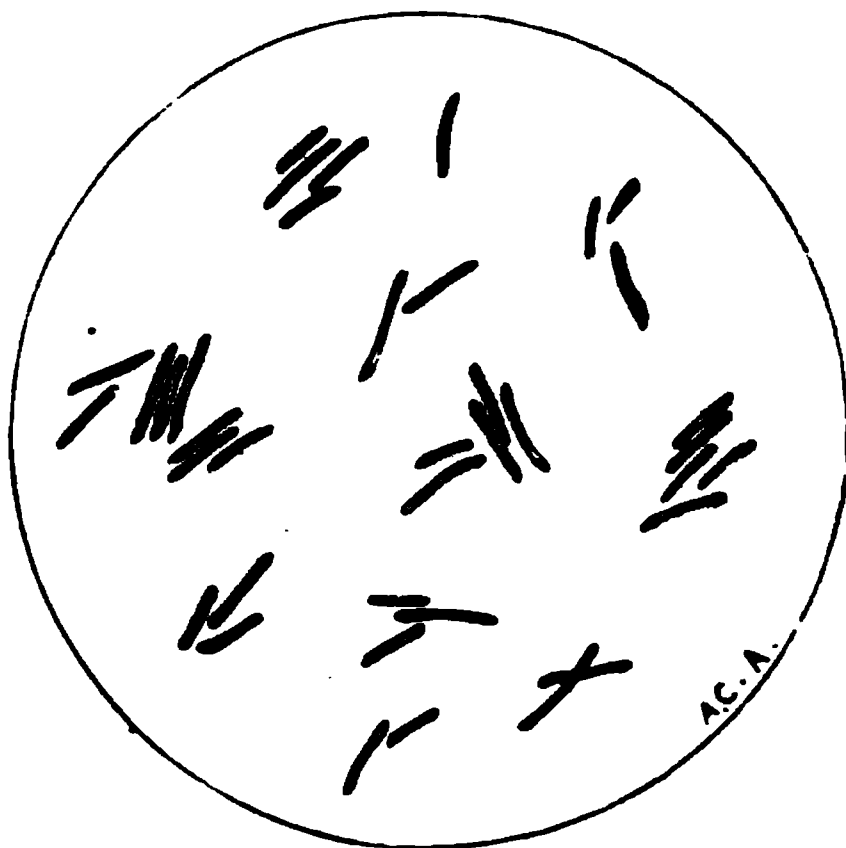
¹ Jour. Med. Research, 1904, xii, 475.

² Zeitschrift für Hygiene und Infektionskrankheiten, 1897, Bd. xxiv.

2 grams, dissolved in 1 litre of boiling distilled water, filtered, and allowed to cool. It is again rinsed in water and examined as a water-mount, or it may be dried and mounted in balsam.

When so treated the diphtheria bacterium appears as faintly stained brown rods, in which from one to three dark-blue granules are to be observed. The dark granules are at one or both poles of the cell, are more or less oval,

FIG. 83



Bacterium diphtheriæ, stained by Neisser's method.

and usually seem to bulge a little beyond the contour of the bacterium in which they are located. (See Fig. 83.) From Neisser's observations and those of others,¹ as well as from personal experience, it seems safe in the vast majority of

¹ Fränkel, Berliner klin. Wochenschrift, 1897, No. 50. Bergey, Publications of the University of Pennsylvania, New Series, 1898, No. 4.

cases to regard all bacteria that do not stain in the way described as distinct from bacterium diphtheriæ.

Blumenthal and Lipskerow¹ decide that the differential method which yields the most satisfactory results consists in the fixation of the preparation for from one-half to two minutes in the following solutions: Pyoktanin (Merck) 0.25 grams, acetic acid (5 per cent.) 100 c.c.; washing in water and counterstaining with a 1 to 1000 solution of vesuvin for one-half minute. By this method the polar granules of bacterium diphtheriæ are stained bluish black, are large, and may be seen in almost all of the organisms. The contour of the darkly stained bacterium diphtheriæ is sharply defined, and it is very easily differentiated from any other organisms that may be present in the preparation.

NOTE.—Prepare cover-slip preparations from the mouth-cavities of healthy individuals and from those having decayed teeth. Do they correspond in any way with those made from diphtheria? Do the same with different forms of sore-throat. Do the peculiarities of any of the organisms suggest those of bacterium diphtheriæ? Wherein is the difference?

In cultures and cover-slips made from both diphtheritic and from innocent sore-throats are any organisms almost constantly present? Which are they, and what are their characteristics?

Which are the predominating organisms in the anginas of scarlet fever?

Do these organisms simulate, in their cultural and morphological peculiarities, any of the different species with which you have been working?

¹ Centralblatt f. Bacteriologie, Bd. xxxviii, p. 359.

Do the diphtheria organisms disappear from the throat with the disappearance of the membrane? How long do they persist? When obtained from the throats of convalescents are they still pathogenic for guinea-pigs?

Prepare a bouillon culture of virulent *bacillus diphtheriæ*; after it has been growing for thirty-six hours at 37°–38° C. inoculate a guinea-pig subcutaneously with about 0.1 c.c. of it. If the animal dies, note carefully the findings at autopsy, especially the distribution of the bacilli. Now add to this culture sufficient pure carbolic acid or trikresol to kill all bacteria in it, and inject under the skin of another guinea-pig varying amounts of the culture so treated, beginning with 0.05 c.c.; determine the minimum fatal dose, and note in which respects the postmortem findings simulate and in which they differ from those of the first animal. Should any of the animals survive the injections of the disinfected culture, note carefully their condition from day to day, particularly any fluctuations in weight. When they have quite recovered inoculate them with living, virulent diphtheria organisms. Do the results correspond with those obtained with guinea-pigs that have never been treated at all? Explain the results.

Diphtheria Antitoxin.—As stated above, the growth of bacterium *diphtheriæ* is accompanied by the elaboration of a poison of remarkable toxicity that is accountable for the constitutional symptoms and pathological lesions by which the disease is characterized. If by appropriate methods this poison (toxin) be separated from the bacteria by which it was formed, it is capable, when injected into susceptible animals, of causing death and practically all the lesions that accompany the disease when due to the invasion of the living bacteria. If, on the contrary, the dose of poison

be so adjusted as to cause only temporary inconvenience and not endanger life, and this dose be injected repeatedly, gradually increasing in size as the animal is able to bear it, after a while a marked tolerance is established, so that the animal may be given many times the amount of the toxin that would otherwise prove fatal—*i. e.*, many times the lethal dose for an animal that had not acquired such a tolerance.

If blood be now drawn from the animal that has become habituated, so to speak, to the diphtheria toxin, and the serum collected from it, we discover several important facts, viz.:

That this serum when mixed with the previously determined lethal dose of the toxin in a test-tube will either neutralize its toxicity or greatly reduce it, according to the amount of serum used.

That if we inject into an animal the determined fatal dose of the toxin, and immediately afterward inject a quantity of the serum, either the animal will not die or the death will be more or less delayed, according to the amount of serum employed.

That if a susceptible animal be inoculated with a living culture of virulent bacterium diphtheriæ, its life may be saved, or its death postponed, by the subsequent injection of the serum; the result depending upon the amount of serum used and the lapse of time between inoculation with the bacteria and injection of the serum.

And, finally, that although this serum has such a marked effect upon the *toxins* of bacterium diphtheriæ in a test-tube or in the animal, and so striking an influence upon the course of infection with the living organisms in the animal, it has little or no effect upon the living bacteria

either in a test-tube or at the site of inoculation in the living animal body.

This serum with which we have been experimenting is the so-called "diphtheria antitoxin" or "antidiphtheritic serum."

For practical purposes, it is obtained from horses, the animals being treated with gradually increasing doses of diphtheria toxin until they are able to withstand enormous multiples of the ordinarily fatal dose. When this point is reached, the protective body—the antitoxin—is present in the blood in such large quantities that the serum may be successfully employed in the treatment of diphtheria in human beings—*i. e.*, as an antidote to the diphtheria toxin that is produced by the growing bacteria in the throat, or elsewhere, and distributed through the body by the circulating fluids.

The Standardization of Diphtheria Antitoxin.—The value of diphtheria antitoxin may be determined according to several different standards. Those that are best known have been proposed by Behring and by Ehrlich.

1. *Behring's Method.*—He designates as a "normal" poison a toxin of which 0.01 c.c. suffices to kill a guinea-pig weighing 250 grams in four days. Of such a normal diphtheria toxin 1 c.c. will be sufficient to kill 100 guinea-pigs weighing 250 grams each, or 25,000 grams in weight of guinea-pigs.

The quantity of antitoxin that is required to just protect 25,000 grams weight of guinea-pigs from the minimum fatal dose of the toxin is called one immunizing unit. If an immune serum contains in 1 c.c. one immunizing unit, it represents a "normal" antitoxin.

To determine the strength of an immune serum, 1 c.c. of

normal toxin is mixed with increasing quantities of the serum, and these mixtures are injected subcutaneously into guinea-pigs; the quantity of the serum which suffices to neutralize that amount of normal toxin—*i. e.*, that keeps the animal alive for four days or longer—contains one immunizing unit.

2. *Ehrlich's Method.*—Ehrlich introduced the use of a standard diphtheria antitoxin in a dry state which contains 1700 immunizing units in each gram. This standard antitoxin, distributed by the Institute for testing serum at Frankfort-on-the-Main, is now being used in a great many places for the standardization of diphtheria antitoxin. A test toxin is prepared, corresponding to this standard antitoxin, and with this toxin the strength of the unknown serum is titrated.

If, for instance, the test toxin is of such a strength that 0.003 c.c. represents the minimum fatal dose for a guinea-pig of 250 grams, then 0.3 c.c. would represent 100 times the minimum fatal dose of toxin, and, according to Ehrlich's standard, an immunity unit is that amount of antitoxic serum which will neutralize 100 times the minimum fatal dose of toxin. In performing the test to estimate the strength of an antitoxic serum, the antitoxin is diluted with sterile water in varying proportions, and a series of guinea-pigs are injected with mixtures of 100 times the minimum fatal dose of the toxin and varying quantities of the diluted antitoxic serum. For this purpose guinea-pigs of approximately 250 grams weight are employed. If, for instance, a guinea-pig receiving 100 times the minimum fatal dose of toxin, and 0.1 c.c. of the diluted antitoxic serum, survives for four days, then 0.1 c.c. of the serum represents an immunity unit of antitoxin.

An antitoxic serum of this strength, therefore, contains 10 times the normal amount of antitoxin, because it contains the immunity unit in only 0.1 c.c.; a normal antitoxin being one in which an immunity unit is contained in one cubic centimeter. Antitoxic serums are frequently of such high degree of potency that they contain from 800 to 1000 immunity units in each cubic centimeter.

CHAPTER XXIV.

**Typhoid Fever—Study of the Organism Concerned in its Production—
Its Morphological, Cultural, and Pathogenic Properties—*Bacillus Coli*
—*Bacillus Paratyphosus*—Its Resemblance to *Bacillus Typhosus*.**

BACILLUS TYPHOSUS.

THE organism seen in the cadavers of typhoid subjects by Eberth (1880–81), and subsequently isolated in pure culture and described by Gaffky (1884), is generally recognized as the exciting factor of typhoid fever. It may be described as follows:

FIG. 84



Bacillus typhosus, from cultures twenty-four hours old, on agar-agar.

FIG. 85

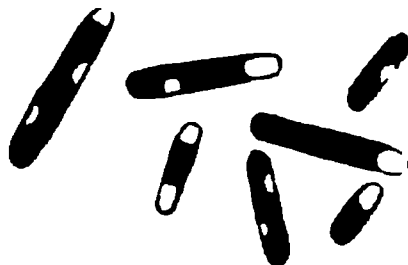
Bacillus typhosus, showing flagella stained by Löffler's method.

Morphology.—It is a bacillus about three times as long as broad, with rounded ends. It may appear at one time as very short ovals, at another time as long threads, and both forms may occur together. Its breadth remains toler-

ably constant. Its morphology presents little that will aid in its identification. (See Fig. 84.) It is actively motile, and when stained by special methods, is seen to possess very delicate locomotive organs in the form of fine, hair-like flagella, attached in large numbers to all parts of its surface. (See Fig. 85.) These flagella are not seen in unstained preparations, nor are they rendered visible by ordinary methods of staining. (See methods for staining flagella.)

Owing to a tendency to retraction of its protoplasm from the cell-envelope and the consequent production of vacuoles in the bacilli, the staining of this organism is frequently

FIG. 86



Diagrammatic representation of retraction of protoplasm, with production of pale points, in *bacillus typhosus*.

more or less irregular. At some points in a single cell marked differences in the intensity of the staining will be seen, and here and there areas quite free from color can commonly be detected. These colorless portions are often so sharply defined that they look as if they had been punched out with a sharp instrument. (See Fig. 86.)

It does not form spores.

Gelatin Plates.—Its growth, when seen in the depths of the medium, presents nothing characteristic, appearing simply as round or oval, finely granular points. On the surface it develops as very superficial, blue-white colonies, with irregular borders. They are a little denser at the

centre than at the periphery. When magnified, the colonies present wrinkles or folds, which give to them, in miniature, the appearance seen in relief maps (Fig. 87). These colonies have sometimes the appearance of flattened pellicles of glass-wool, and usually a pearl-like lustre.

Agar-agar.—On agar-agar the colonies present nothing typical.

Stab-cultures.—In stab-cultures the growth is mostly on the surface, there being only a very limited development down the track made by the needle. The surface-growth has the same appearance in general as that given for the colonies.

FIG. 87

Colony of bacillus typhosus on gelatin

Potato.—The growth on potato is usually described as luxuriant but invisible, making its presence evident only by the production of a slight increase of moisture at the inoculated point, and by a limited resistance offered to a needle when it is scraped across the track of growth. While this is so in many cases, yet it cannot be considered as invariable, for at times this organism develops more or less visibly on potato.

Potato-gelatin.—The growth is similar to that upon ordinary nutrient gelatin.

Milk.—It does not cause coagulation when grown in sterilized milk.

Bouillon.—It causes uniform clouding of the bouillon and brings about a slightly acid reaction.

Indol Formation.—It is customary to regard this organism as devoid of the power to form indol; in fact, this has hitherto been considered one of its important differential peculiarities, and by the usual methods of cultivation and testing the indol reaction is not observed in cultures. It has been shown, however, by Peckham, that by repeated transplantation, at short intervals, into either Dunham's peptone solution, or, preferably, a freshly prepared alkali-tryptone solution, made from tryptonized beef-muscle, that the indol-producing function may be induced in the genuine typhoid bacillus obtained directly from the spleens of typhoid cadavers.¹

It does not produce gaseous fermentation. On lactose-litmus-agar-agar it grows as pale-blue colonies, causing no reddening of the surrounding medium; though if glucose be substituted for lactose, both the colonies and the surrounding medium may become red. In the fermentation-tube, in glucose or lactose bouillon, no evolution of gas as a result of fermentation occurs.

It grows at any temperature between 20° and 38° C., though more favorably at the latter point. It is very sensitive to high temperatures, being killed by an exposure of ten minutes to 60° C., and in a much shorter time to slightly higher temperatures.

It does not liquefy gelatin.

It grows both with and without oxygen.

It does not grow rapidly. '

¹ A. W. Peckham, The Influence of Environment Upon the Biological Functions of the Colon Group of Bacilli, *Journal of Experimental Medicine*, 1897, vol. ii.

Presence in Tissues.—In patients suffering from typhoid fever the organism has been found during life by the application of appropriate culture methods in the blood, urine, and feces, and at autopsies in the tissues of the spleen, liver, kidneys, intestinal lymphatic glands, and intestines. It is not easy to demonstrate this organism in tissues unless it is present in large numbers. The manipulations to which the sections are subjected in being mounted often rob the bacilli of their stain, and render them invisible, or nearly so. If, however, sections be stained in the carbol-fuchsin or the alkaline methylene-blue solution, either at the ordinary temperature of the room or at a higher temperature (40° to 45° C.), then washed in absolute alcohol, and cleared in xylol¹ and mounted in xylol balsam, the bacilli (particularly if the tissues be the liver and spleen) can readily be detected, massed together in clumps.

In searching for the typhoid bacilli in tissues this peculiar disposition in clumps must always be borne in mind, otherwise much labor will be expended in vain. In tissues the typhoid bacilli are not scattered about as are the organisms in certain other conditions—septicemia, for instance; they are not regularly distributed along the course of the lymphatics or capillaries, but appear in small masses through the organs, and it is for these agglutinations that one should search. This peculiar clumping of the typhoid bacilli in the tissues cannot be satisfactorily explained, unless it be due to the specific agglutinating influence that typhoid blood has upon the typhoid bacillus, a phenomenon that is readily demonstrable in the test-tube or under the microscope. In other words, may it not be simply the result of an intracapillary “Widal reaction”? (See Widal Reaction.)

¹ Do not clarify with oil of cloves. It is too active as a decolorizer.

When the section is prepared for examination, if it be gone over with a *low-power* objective, one will notice at irregular intervals little masses that look in every respect like particles of staining-matter which have been precipitated upon the section at that point. When these masses are examined with a higher power objective they will be found to consist of small ovals or short rods so closely packed that the individuals composing the clump can often be seen only at the extreme periphery of the mass. This is the characteristic appearance of the typhoid organism in tissues, to which allusion has just been made. The little masses are usually in the neighborhood of a capillary.

Isolation of *Bacillus Typhosus* from Cadavers.—The spleen of a patient dead of typhoid fever is the most reliable source from which to obtain cultures of the typhoid bacillus for study. But it must always be remembered that the same channels through which the typhoid bacillus gains access to this viscus are likewise open to other organisms present in the intestines, and for this reason *bacillus coli*, a normal inhabitant of the colon, may also be found in this locality.

Result of Inoculation into Lower Animals.—A great many experiments have been made in a variety of ways with the view of reproducing the pathological conditions of this disease, as seen in man, in the tissues of lower animals, but with practically no success. From the time of its discovery up to within a comparatively recent date there was an almost continuous controversy concerning the infective properties of *bacillus typhosus* for animals. By some it was held that the effects of its introduction into animals were manifestly of toxic¹ origin, while others regarded them as evidences of

¹ Toxic—poisonous results not necessarily accompanied by the growth of organisms throughout the tissues.

genuine infection.¹ These diversities of opinion are hardly surprising when we remember that animals never suffer naturally from typhoid fever, and therefore offer many obstacles to its faithful reproduction, and that the vigor of this organism when cultivated from various sources is liable to a wide range of fluctuation. Numerous investigations lead to the belief that the poison peculiar to this organism is so intimately bound up with its protoplasmic structure as to make its separation difficult, if not impossible. However, by the use of dead cultures (*i. e.*, cultures of well developed organisms destroyed by heat) results are obtained that leave no doubt that the clinical symptoms and pathological changes seen in man and in animals under experiment are referable to a specific intoxication, and, as a rule, the only effects that follow the introduction of this organism into animals are referable to the intoxicating action of the materials used. In fact, the results of modern investigations have placed bacillus typhosus in the category of endotoxin producers, and through the use of the toxins (not pure, but mixed with other substances in the culture media) produced by it animals have been rendered immune from otherwise fatal doses of highly toxic cultures. The serum of such animals has also been shown to possess a certain degree of immunizing power.²

Because of the variations in the morphology and physiology of this organism, and because of the difficulty experienced in efforts to reproduce in lower animals the condition found in the human subject, our knowledge of typhoid fever, though fairly accurate in many respects, is, never-

¹ Infective or septic—poisoning of the tissues as a result of the growth of bacteria within them.

² Pfeiffer and Kolle, *Zeitschrift für Hygiene und Infektionskrankheiten* 1896, Bd. xxi, S. 208.

theless, in certain essential details relating to its causation, very far from satisfying.

A number of other organisms appear botanically to be closely related to the typhoid bacillus, and under the available culture methods for studying them they so closely simulate it that the difficulty of identifying this organism is sometimes very great. In addition the variability constantly seen in pure cultures of the typhoid bacillus itself in no way renders the task more simple.

For example, the morphology of the typhoid bacillus is conspicuously inconstant; its growth on potato which was formerly considered unique, may, with the same stock, at one time be the typical invisible development, at another it is easily to be seen with the naked eye; and the change of reaction which it is said to produce in bouillon is sometimes much more intense than at others. The indol-producing function, hitherto regarded as absent from this organism, is now known to be occasionally demonstrable by ordinary methods, and frequently by special methods of cultivation (Peckham, *l. c.*). The only properties exhibited by it under the usual conditions of cultivation that may be said to be constant are its motility; its inability to cause gaseous fermentation of glucose, lactose, or saccharose; its incapacity for coagulating milk; and its growth on gelatin plates but there are other bacilli which possess these same characteristics to a degree that renders their differentiation from the typhoid organism often a matter that requires the careful application of all the different tests.

The Agglutination Reaction.—The nearest approach to a trustworthy means of identification is the specific reaction of typhoid bacilli with the blood of typhoid subjects. When typhoid bacilli are brought in contact with the blood-serum

from human beings sick of typhoid fever, or from animals that have survived inoculation with cultures of this organism, there occurs a peculiar alteration in the relation of the organisms to one another in the fluid. As ordinarily seen in a hanging drop of bouillon, the typhoid bacilli appear as single, actively motile cells; when to such a drop a little dilute serum from a case of typhoid fever is added the motility of the bacteria gradually lessens and finally ceases, and they then congregate, "agglutinate" in larger and smaller clumps, or if one add to 4 or 5 c.c. of a twenty-four-hour-old bouillon culture of typhoid bacilli in a narrow test-tube about eight drops of serum from a case of typhoid fever and maintain this mixture at body temperature the normally clouded culture will be seen after a few hours to have undergone a change; instead of a diffuse clouding it is clear and flocculent masses of the bacteria that have agglutinated together as a result of the specific action of the serum used will be scattered about in it.

For the hanging-drop test, sufficient serum may be obtained from a needle-prick in the finger, while for the test-tube reaction a larger amount is needed; this may be obtained from blood drawn from a superficial vein by means of a hypodermic syringe, or from the cleansed skin by a wet-cup, or, better still, from a small cantharides or ammonia blister.

It is proper to state, however, that occasionally cultures of genuine typhoid bacilli are encountered that do not respond to this peculiar influence of typhoid blood, even though the blood be tested at different stages of the disease, and even though it may cause the characteristic cessation of motion and clumping with other cultures of this organism upon which it is tried.

“Widal’s Reaction.”—When employed conversely—*i. e.*, for deciding if the serum used is from a case of typhoid fever or not—the reaction constitutes “Widal’s serum diagnosis of typhoid fever.” In beginning these tests it is often necessary to try several cultures of genuine typhoid bacilli from different sources and of varying degrees of vitality, before a strain is procured that reacts conspicuously and quickly with genuine typhoid serum.

WIDAL’S REACTION WITH DRIED BLOOD.—This reaction can also be obtained with redissolved dried blood—*i. e.*, by the Johnston method: a drop of the blood to be tested, obtained by a needle-prick in the cleansed finger or lobe of the ear, is collected on a bit of clean, unglazed paper and allowed to dry. The paper is then folded, kept free from contamination, and taken to the laboratory. With a medium-size platinum-wire loop a drop of sterile bouillon, water, or physiological salt solution is gently rubbed upon the loop of dried blood until the contents of the loop are of a dark amber color; this is then mixed with a drop of bouillon culture of typhoid bacilli on a cover-glass, which is mounted upon the hollow-ground slide as a hanging drop, when the effect of the diluted blood upon the culture can be observed with the microscope. The reaction, if positive, should occur within a half hour. Many object to this method because it is impossible accurately to dilute the blood by the plan used. A number of tests have shown us that preparations made in this way correspond roughly with a fresh-blood dilution of from 1 : 15 to 1 : 20, as determined by the hemoglobinometer. In a small number of cases in which parallel tests were made with this and with fresh fluid serum the results were concordant. We are inclined to the opinion, however, that in doubtful cases, in

which all the available clinical evidence is opposed to either the positive or negative results of the test, the difficulty is much more certainly cleared away by the use of *highly* dilute and *exactly* diluted fresh serum than by this method. Competent observers are of the opinion that in all such cases the quantity of serum in the hanging drop should be decreased until it is present in the proportion of from 1 : 50 to 1 : 60, and that, if after exposure to this dilution for two hours the bacilli are still motile and not clumped together or the reaction is deficient in only one or the other of these peculiarities, the case from which the serum was obtained may be safely regarded as not typhoid fever, or if typhoid the examination was not made at a time when agglutinin was present in demonstrable quantities in the circulating blood.

Experience with both the dry-blood and the fresh serum methods at the Municipal Laboratory of Philadelphia in more than 12,000 examinations from about 10,000 febrile conditions, lead us to regard the culture used as one of the most important factors in the test. After deciding upon the most suitable culture for the reaction—and it is often necessary to try a great number from various sources—we have adopted the plan of daily transplanting the culture into fresh bouillon and keeping it at a temperature rarely above 20° or 22° C. The bacilli grown under these circumstances are usually somewhat longer than when cultivated at higher temperature, and they exhibit a regular, gliding motility that renders it more easy to follow the individual cells under the microscope than when they possess the usual active, darting motion.

In the group of cases examined by us by the dry-blood method, including typhoid and other febrile conditions

there is a discrepancy between the clinical and the laboratory diagnosis in from 2 to 3 per cent. of the cases examined.

In the hands of all who have carefully employed the Widal reaction for the diagnosis of typhoid fever the results are reported to have been almost uniformly satisfactory. In the great majority of cases the reaction is, so far as experience indicates, specific—*i. e.*, a typical reaction does not occur between typhoid serum or blood and organisms other than the typhoid bacillus, nor between the typhoid bacillus and serums other than those of typhoid fever. There are, however, confusing reactions—so-called pseudoreactions—in which more or less clumping of the bacilli and a diminution of motion, without complete cessation, are observed. These reactions have been seen to occur with normal blood and with blood from other febrile conditions. It is said by Johnston and McTaggart¹ that they can be prevented if cultures of *just the proper degree of vitality* are employed; and this corresponds with the results of a fairly wide personal experience with the test.

In the light of present experience it is fair presumptive evidence that the serum is from a case of typhoid fever when unmistakable agglutination and cessation of motion are seen in from fifteen to twenty minutes after typhoid bacilli are mixed with the serum of a conspicuous febrile condition.

The blood of certain animals, as well as a number of chemical substances, such as corrosive sublimate, alcohol, salicylic acid, resorcin, and safranin in high dilution, cause agglutination of the typhoid bacilli; but the reaction is not specific, for in most cases they have the same effect on other motile bacilli.

¹ Montreal Medical Journal, March, 1897.

Drinking Water.—All the points with regard to morphologic and biologic characters of bacillus typhosus, and of the organisms closely resembling it, should be borne in mind in the examination of drinking-water supposed to be contaminated by typhoid dejections, for the organisms which most closely approach the typhoid bacillus in growth and morphology are just those organisms which would appear in water contaminated from cesspools—*i. e.*, the organisms constantly found in the normal intestinal tract. Even in the stools of typhoid-fever patients the presence of these normal inhabitants of the intestinal tract renders the isolation of the typhoid organisms somewhat troublesome.

Methods of Isolating the Typhoid Bacillus.—From the foregoing it is obvious that bacillus typhosus is so variable in many of its biological peculiarities, and is so closely simulated in certain respects by a group of other organisms to which it appears to be botanically related, that its identification, especially outside the infected body, is a matter of considerable difficulty and uncertainty. For these reasons many efforts have been made to discover specific cultural reactions for the organism, and with this end in view many methods have been devised for its isolation from water, feces, sewage, and other matters believed to contain it. None of them, however, have given general satisfaction, and many have proved wholly untrustworthy.

In deciding upon a suitable routine these are several points that should be borne in mind:

(a) As bacillus typhosus when present in water, feces, soil, milk, etc., is always numerically in the minority, as compared with other organisms, it is desirable to employ a method that will encourage its multiplication without at the same time favoring the same rate of multiplication

by other organisms present, that is to say, to use an “enriching medium;” (b) and to possess a method that will make comparatively simple the isolation or separation of the typhoid bacilli, after “enrichment,” from the other organisms with which it is associated. With these objects in mind a routine that gives very general satisfaction is as follows:

Enriching Media.—For this purpose ox bile and “brilliant green” have been found to favor the growth of typhoid bacilli, and to be less favorable to the growth of other organisms associated with it; consequently if a bit of typhoid feces or a portion of infected water or milk be mixed with either of these media and kept at suitable temperature for a time, the result will be a more conspicuous growth of bacillus typhosus than of the other organisms.

Two forms of ox bile may be employed:

(1) Pure fresh bile direct from the gall-bladder of a freshly-slaughtered ox, or (2) a solution of peptone and dried ox bile of the following proportions:

Dried ox bile	10 parts
Peptone	1 part
Water	100 parts

In either event test-tubes or flasks are filled with convenient amounts and sterilized; after which they are ready for inoculation with the mixture suspected of containing the typhoid bacillus. After inoculation they are kept at body temperature for about twenty-four hours, when plates may be made with the differential media to be described below.

Instead of the ox bile the aniline dye known as “brilliant green” may be employed. This substance suppresses to

some extent the growth of organisms other than bacillus typhosus, particularly those of the colon group. It is used in the following manner: To test-tubes containing a known amount (8 to 10 c.c.) of peptone solution, "brilliant green" is added in varying amounts so as to have a series of solutions ranging in strength from one part of the green to 500,000, to one part to 100,000 of the peptone solution. A convenient stock solution of the "brilliant green" is 1 : 1000 in water. From this such amounts are added to the tubes of peptone solution as will give the desired series of dilutions. The tubes of peptone solution should have been sterilized before the green is added. When ready, one adds to each of these tubes an amount of the substance under consideration: if it be feces—a moderate loopful may be broken up in 1 c.c. of bouillon and one or two loopfuls of this used; if it be water or milk from 0.1 to 0.3 c.c. The amount best suited must be determined by experiment.

When inoculated the tubes are kept at body temperature for from eighteen to twenty-four hours, when they are ready for the "differential" or "selective" plating.

The enriching media should be free of sugar.

In the process of plating, specially prepared selective media are used that aim to render evident to the naked eye distinguishing differences between the colonies of bacillus typhosus and those of other confusing organisms. Of a number of special media employed for this purpose two have proved very satisfactory—notably that recommended by Drigalski and Conradi, and that by Endo.

METHOD OF V. DRIGALSKI AND CONRADI.¹—This method aims to separate bacillus typhosus from bacillus coli on the basis of their fermenting properties, in such a manner as not

¹ Zeitschrift für Hygiene, 1902, Bd. xxxix, p. 288.

to hinder the growth of *bacillus typhosus*, but rather to make the conditions for its growth as favorable as possible.

The authors give the following directions for the preparation of their culture medium:

a. Preparation of agar: 1500 grams of finely chopped beef are placed in two litres of water and set aside for twenty-four hours. This meat infusion is then boiled for one hour, filtered, and 20 grams of Witte's peptone, 20 grams of nutrose, and 10 grams of sodium chloride are added and again boiled for an hour, filtered, and 60 grams of agar-agar are added, boiled for three hours (or one hour in the autoclave), rendered slightly alkaline to litmus-paper, filtered, and boiled for one-half hour.

b. Litmus solution: (Litmus solution according to Kubel and Tiemann) 260 c.c., boil ten minutes, add 30 grams chemically pure lactose, boil fifteen minutes.

c. The hot litmus-lactose solution is added to the hot nutritive agar, thoroughly mixed, and the alkaline reaction is again restored. To this medium is then added 4 c.c. of a hot sterile solution of 10 per cent. water-free sodium carbonate, 20 c.c. of freshly prepared solution of 0.1 gram crystal violet (Höchst) in 100 c.c. of warm sterile distilled water.

One now has a meat-infusion-peptone-nutrose-agar with 13 per cent. of litmus solution and 0.01 per thousand crystal violet. It becomes very hard on solidifying, without becoming too dry. Plates are poured of this material and held in readiness for some time, and the remainder of the medium is preserved in flasks in portions of 200 c.c. each.

If the lactose is boiled for a longer time than directed it is reduced, with an acid reaction of the culture medium, and the content in lactose falls below the required quantity,

and the alteration in the color of the colon colonies appears too early. For this reason it is also necessary to liquefy the agar as quickly as possible in pouring plates from the agar medium stored in flasks.

In employing this culture medium it is necessary to have a uniform suspension of a portion of the material to be examined and to make a series of plate inoculations from this suspension *by smearing carefully the material under consideration over the surface of the medium in the plates*, a sterile platinum spatula or a sterile bent glass rod being used for the purpose.

After fourteen to sixteen hours at 37° C., and still better after twenty to twenty-four hours, the cultures are readily differentiated:

a. Bacillus Coli: All cultures of true colon that have been examined form colonies of 2 to 6 or more millimeters in diameter, of reddish color and translucent. In each intestinal evacuation there are usually several varieties of colon colonies which differ according to their size and texture, translucency, and the intensity of the alteration of the color which they bring about. Many colon colonies are bright red, some are cloudy, and others are quite opaque, dark-wine red in color, while still others form large colonies which are surrounded by a red halo.

b. Bacillus Typhosus: The colonies have a diameter of 1 to 3 millimeters, rarely larger. Their color is blue, with a tendency toward violet. In structure they are glistening, with a single contour, somewhat of the nature of a dew drop. Only in isolated instances is the colony larger and more cloudy in appearance.

The Endo Media.—(Modification of Kendall and Day.)
Prepare the following:

(a) Water	1000 c.c.
Powdered agar-agar	15 grams
Peptone (Witte)	10 grams
Meat Extract (Liebig)	3 grams

Heat until the agar-agar is dissolved, keeping the mass to 100 c.c. volume by addition of water. This should require about an hour over the flame, or less if the mass be dissolved in the autoclave. Render just alkaline to litmus by the addition of deci-normal sodium hydroxide solution. Filter and decant to flasks containing 100 c.c. each. Sterilize.

(b) Prepare a 10 per cent. solution of fuchsin in 96 per cent. alcohol.

(c) Prepare a 10 per cent. solution of sodium sulphite in water.

For the making of the plates mix 1 c.c. of (b) with 10 c.c. of (c) and heat in the steam sterilizer (100° C.) for 20 minutes. This decolorizes the fuchsin. To each 100 c.c. of the agar prepared as (a), add 1 per cent. of chemically pure lactose and heat in the steam sterilizer at 100° C. until the agar-agar is completely liquefied and the lactose dissolved. To each 100 c.c. of this lactose-agar add 1 c.c. of the decolorized fuchsin solution, mix thoroughly and while still fluid and warm, pour into sterile Petri dishes; sufficient in each dish to give a layer of from 3 to 5 mm. depth. Place these dishes, with the covers removed, in the incubator until the agar-agar has set; this will require about 30 minutes. They are then ready for inoculation. The plates are now inoculated by spreading evenly over the surface small quantities from the primary "enriching" cultures. This is best done by the use of a bent glass rod that has been sterilized in the flame and allowed to cool.

If typhoid bacilli be present they develop as tiny, transparent, practically colorless colonies of from 1 to 2 mm.

in diameter. Colonies of the colon or para-colon group appear as larger, denser pink or red masses and cause a reddening of the medium about them.

All small, transparent, colorless colonies, *i. e.*, those suggestive of bacillus typhosus are to be isolated in pure culture and identified by the usual procedures.

Precipitation Method of Ficker.¹—Two liters of the water to be examined are placed in a narrow sterile glass cylinder and rendered alkaline with 8 c.c. of 10 per cent. sodium carbonate solution, and afterward 7 c.c. of a 10 per cent. sulphate of iron solution are added and mixed with the water by means of a sterile glass rod. The cylinder is then placed in the ice-chest. Precipitation is complete in two to three hours. The overstanding water is syphoned off, and the precipitate or portions thereof are poured into sterile test-tubes. To this precipitate is now added about a half volume of a 25 per cent. solution of neutral potassium tartrate. The test-tube is closed with a sterile rubber cork and the mixture thoroughly agitated, whereby the precipitate is completely dissolved. With a sterile pipette one part of this fluid is mixed in a test-tube with two parts of sterile bouillon, and this mixture is distributed over a series of Drigalski-Conradi plates. Ficker advises when possible the use of a centrifuge for the separation of the precipitate, as he believes the results are likely to be more satisfactory.

Prophylactic Vaccination.—That typhoid fever may be prevented by vaccination is an accomplished fact. Experience gathered during the past few years by all civilized governments, notably those of England, France, Germany and this country is unanimous in support of this statement.

No argument could be more convincing than the results

¹ Hygienische Rundschau, 1904, Bd. xiv, S. 7.

obtained through the vaccinations practiced in the United States Army and Navy, where the procedure is now compulsory. The following abstract from one of the several excellent reports submitted by Major Russell of the U. S. Army Medical Corps, suffices to illustrate the protective value of antityphoid vaccination:

In 1898, during the Spanish-American War, when no preventive vaccination was practised, there were assembled at Jacksonville, Florida, 10,759 troops, among whom there were *certainly* 1729 cases of typhoid fever, and including those cases that were *probably* typhoid fever, this figure is increased to 2,693 cases with 248 deaths. Contrast that with the following:

In 1911 there were assembled for maneuvers along the Mexican frontier about 20,000 United States troops. All were vaccinated against typhoid fever; with the result that after four months in camps (about the same time as the men remained in the Jacksonville camp) there developed *one case* of typhoid fever. This case did not prove fatal. It should be said that the disease was known to exist among residents in the immediate vicinity of this camp and that the soldiers were allowed free access to the infected districts.

By the adoption of compulsory vaccination in the Army, typhoid fever has been practically eliminated. For the entire United States the typhoid mortality for the year 1913 was at the rate of 12.7 per 100,000, while for the entire army it was 0 per 100,000.

It is needless to pursue the argument further; though it should be said that the vaccination is harmless to the individual.

Major Whitmore of the Medical Corps of the United

States Army states that of 130,000 adults vaccinated, 97 per cent. gave no disagreeable reaction.

Major Russell has also shown by a very careful study that children under five years of age may be safely vaccinated if appropriate doses of the vaccine be employed.

The Vaccine.—The agent used in vaccination is typhoid bacilli that have been killed by heat. In some instances living, sensitized typhoid bacilli have been employed with good results, but as the bulk of experience has been obtained with the dead cultures and as this is much the more simple procedure it is probable that it is the method that will be generally adopted.

The vaccine is prepared as follows: A proven culture of bacillus typhosus is grown on nutrient agar-agar at body temperature for eighteen to twenty hours. The growth is then carefully washed from the surface with a small quantity of sterile physiological salt solution. This emulsion is then heated in a water bath to 53° C. for one hour, after which it is diluted with sterile salt solution to a point at which a billion bacilli are contained in a cubic centimeter of the emulsion. Finally tricresol in the proportion of 0.25 per cent. is added as a preservative. Before using such vaccine its safety: *i. e.*, its freedom from objectionable qualities, especially from the germs of tetanus, is invariably tested, as is also its efficiency in calling forth the customary reactions of intoxication and resistance. These tests are made upon such sensitive reagents as mice, guinea-pigs, and rabbits.

The vaccination consists in the subcutaneous injection of a volume of emulsion equivalent to 500 million bacilli followed on the tenth and twentieth days with doses equivalent to 1000 million bacilli, that is to say the first dose is

0.5 c.c. of the above-mentioned emulsion, while the second and third doses are 1 c.c. each.

As a rule the injections—particularly the primary one—are followed by a red, tender, swollen area at the site of puncture. This may be accompanied by headache, fever, general malaise and sometimes by a chill with vomiting or diarrhea. In the majority of individuals the reactions are mild and disappear in from thirty-six to forty-eight hours. In from one to three persons out of every thousand vaccinated the reaction may be severe, though they are not dangerous. No ill effects of a permanent nature have thus far been noted in about 130,000 persons who have been vaccinated in this country, nor have the vaccinations been seen to influence unfavorably the course of other diseases from which the individual may be suffering.

It should be needless to say that strict aseptic precautions are to be taken in performing the operation. The resistance that is, excited by the vaccination is an “active immunity”—that is it is an immunity identical in nature with that acquired by an individual who has recovered from an attack of typhoid fever. In so far as can be stated now, however, the immunity is not permanent. All indications point to its gradual diminution and possible disappearance often in two to three years, so that revaccination after the lapse of this time is advisable.

NOTE.—Obtain a pure culture of typhoid bacilli, and from this make inoculations upon a series of potatoes of different ages and from different sources. Do they all grow alike?

Before sterilizing render another lot of potatoes slightly acid with a few drops of very dilute acetic acid; render

others very slightly alkaline with dilute caustic soda. Are any differences in the growths noticeable?

Make a series of twelve tubes of peptone solution to which rosolic acid has been added. Inoculate them all with as nearly the same amount of material as possible (one loopful from a bouillon culture into each tube); place them all in the incubator. Is the color-change, as compared with that of the control-tube, the same in all cases?

Compare the morphology of cultures of the same age on gelatin, agar-agar, and potato.

Select a culture in which the vacuolations are quite marked. Examine this culture unstained. Do the organisms look as if they contained spores? How would you demonstrate that the vacuolations are not spores? What is the crucial test for spores?

Obtain from normal feces a pure culture of the commonest organism present. Write a full description of it. Now make parallel cultures of this organism and of the typhoid bacillus on all the different media? How do they differ? In what respects are they similar?

BACILLUS COLI (ESCHERICH), MIGULA, 1900.

SYNONYMS: *Bacillus neapolitanus*, Emmerich, 1884; *Bacillus pyogenes foetidus*, Passet, 1885; Emmerich's bacillus, Eisenberg, 1886; *Bacterium coli commune*, Escherich, 1886.

This organism was discovered by Escherich, in 1886, in the intestinal discharges of milk-fed infants. It has since been demonstrated to be a constant inhabitant of the intestines of man and domestic animals, and is, therefore, considered a commensal species.

For a time after its discovery it was considered of but

little importance and attracted attention only because of its resemblance, in certain respects, to the bacillus of typhoid fever, with which it was occasionally confounded. In this particular it still serves as a subject for study. Some have even gone so far as to regard them as but varieties of one and the same species, though in the present state of our knowledge this is an assumption for which as yet there are not sufficient grounds. That they possess in common certain general points of resemblance and often approach one another in some of their biological peculiarities is true; but, as we shall learn, they each possess peculiarities which, when considered together, render their differentiation from one another a matter of but little difficulty.

With the wider application of bacteriological methods to the study of pathological processes it was occasionally observed that, under favorable circumstances, *bacillus coli* disseminated from its normal habitat and appeared in remote organs, often associated with diseased conditions. This was at first considered of but little importance, and its presence in these localities was viewed as accidental. Its repeated appearance, however, in different organs of the body and the frequency of its association with pathological conditions, ultimately attracted attention to it, and in consequence a great deal has been written concerning the possible pathogenic nature of this organism.

The fact that it is a commensal species, always intimately associated with certain of our life-processes, together with the fact that it is known to appear in organs other than that in which it is normally located, and that its occurrence in diseased conditions is not rare, justifies the opinion that it is one of the most important of the micro-organisms with which we have to deal.

While not generally considered a pathogenic organism, there is, nevertheless, sufficient evidence to warrant the statement that under favorable conditions of reduced vitality on the part of the animal tissues, this organism may assume pathogenic properties, so that its presence in diseased conditions is not always to be considered as accidental, though this is frequently the case.

The morphological and cultural peculiarities of *bacillus coli* are as follows:

MORPHOLOGY.—In shape it is a rod with rounded ends, sometimes so short as to appear almost spherical, while again it is seen as very much longer threads. Often both forms are associated in the same culture. It may occur as single cells, or as pairs joined end to end.

It has no peculiar morphological features that can aid in its identification. It is usually said to be motile, and undoubtedly is motile in the majority of cases; but its movements are at times so sluggish that a positive opinion is often difficult.

By Löffler's method of staining, flagella can be demonstrated, though usually not in such numbers as are seen to occur on the typhoid fever bacillus.

Cultural Characteristics.—It grows both with and without free oxygen.

On the surface of gelatin its colonies appear as small, dry, irregular, flat, blue-white points that are commonly somewhat dentated or notched at the margin. They are a trifle denser at the centre than at the periphery, and are often marked at or near the middle by an oval or round nucleus-like mass—the original colony from which the layer on the surface developed. When located in the depths of the gelatin, and examined with a low-power lens, they are at

first seen to be finely granular and of a very pale greenish-yellow color; later they become denser, darker, and much more markedly granular; in shape they are round, oval, and lozenge-like. When the surface colonies are viewed under a low power of the microscope they present essentially the same appearance as that given for the colonies of the bacillus of typhoid fever, viz., they resemble flattened pellicles of glass-wool, or patches of finely ground colorless glass. Colonies of this organism on gelatin are frequently encountered that cannot be distinguished from those resulting from the growth of *bacillus typhosus*; although, as a rule, their growth is a little more luxuriant.

In stab- and smear-cultures on gelatin the surface-growth is flat, dry, and blue-white or pearl color. Limited growth occurs along the track of the needle in the depths of the gelatin. As the culture becomes older the gelatin round about the surface-growth may gradually lose its transparency and become cloudy, often quite opaque. In still older cultures small root- or branch-like projections from the surface-growth into the gelatin are sometimes seen. At times these may be of a distinctly crystalline appearance.

It does not cause liquefaction of gelatin.

Its growth on nutrient agar-agar and on blood-serum is luxuriant, but not characteristic.

In bouillon it causes diffuse clouding with sedimentation. In some bouillon cultures an attempt at pellicle-formation on the surface may be seen, but this is exceptional. In old bouillon cultures the reaction becomes alkaline and a decided fecal odor may be detected.

Its growth on potato is rapid and voluminous, appearing after twenty-four to thirty-six hours in the incubator as a

more or less lobulated layer of a drab, dark-cream, or brownish-yellow color.

In neutral milk containing a little litmus tincture the blue color is changed to red after from eighteen to twenty-four hours in the incubator, and, in addition, the majority of cultures cause firm coagulation of the casein in about thirty-six hours, though frequently this takes longer. *Very rarely* the litmus may indicate the production of acid and no coagulation occur.

In media containing glucose it grows rapidly and causes active fermentation, with liberation of carbonic acid and hydrogen. If cultivated in solid media to which glucose (2 per cent.) has been added, the gas-formation is recognized by the appearance of numerous bubbles along and about the points of growth. If cultivated in fluid media, also containing glucose, in the fermentation-tube, evidence of fermentation is given by the collection of gas in the closed arm of the tube.

On lactose-litmus-agar-agar its colonies are pink and the color of the surrounding medium is changed from blue to red.

In Dunham's peptone solution it produces indol in from forty-eight to seventy-two hours.

It stains with the ordinary aniline dyes. It is decolorized when treated by the method of Gram.

By comparing what has been said of *bacillus typhosus* and of *bacillus coli* it will be seen that, while they simulate each other in certain respects, they nevertheless possess individual characteristics by which they may readily be differentiated. The least variable of the differential points are:

1. Motility of *bacillus typhosus* is much more conspicuous, as a rule, than is that of *bacillus coli*.

2. On gelatin, colonies of the typhoid bacillus develop more slowly than do those of the colon bacillus.

3. On potato, the growth of the typhoid bacillus is usually invisible (though not always); while that of the colon bacillus is rapid, luxuriant, and always visible.

4. The typhoid bacillus does not cause coagulation of milk with acid reaction. The colon bacillus does this in from thirty-six to forty-eight hours in the incubator.

5. The typhoid bacillus never causes fermentation, with liberation of gas, in media containing glucose, lactose, or saccharose. The colon bacillus is conspicuous for its power of causing gaseous fermentation in such solutions.

6. In nutrient agar-agar or gelatin containing lactose and litmus tincture, and of a slightly alkaline reaction, the color of the colonies of typhoid bacillus is pale blue, and there is no reddening of the surrounding medium; while colonies of the colon bacillus are pink and the medium round about them becomes red.

7. The typhoid bacillus does not, *as a rule*, possess the property of producing indol in solutions of peptone; the growth of the colon bacillus in these solutions is accompanied by the production of indol in from forty-eight to seventy-two hours at 37° to 38° C.

Animal Inoculations.—As with the bacillus of typhoid fever, the results of inoculation of animals with cultures of this organism cannot be safely predicted. According to numerous observers the effects that do appear are in most instances to be attributed to the toxic rather than to the infective properties of the culture used.

When introduced into the subcutaneous tissues of mice it has no effect, while similar inoculations of guinea-pigs are sometimes (not always) followed by abscess-formation

at the point of operation, or by alterations very similar to those produced by intravascular inoculation, viz., death in less than twenty-four hours, accompanied by redness of the peritoneum and marked hyperemia and ecchymoses of the small intestine, together with swelling of Peyer's patches. The cecum and colon may remain unchanged or present enlarged follicles. There may or may not be an accumulation of fluid in the abdominal cavity; but peritonitis is rarely present. The small intestine may contain bloody mucus.

Intravenous inoculation of rabbits may be followed by similar changes, with often the occurrence of diarrhea before death, which may, in the acute cases, result in from three to forty hours. In another group of cases acute fatal intoxication does not result, and the animal lives for weeks or months, dying ultimately of what appears to be the effects of a slow or chronic form of infection. For a few hours after inoculation these animals present no marked symptoms; exceptionally, somnolence and diarrhea have been observed at this period, indicating acute intoxication from which the animal has recovered. The affection is unattended by fever. The most marked symptom is loss of weight. This is usually progressive from the first or second day after inoculation, with slight fluctuations until death.

At autopsy the animal is found to be emaciated. The subcutaneous tissues and the muscles appear pale and dry. The serous cavities, particularly the pericardial, may contain an excess of serum. The viscera are anemic. The spleen is small, thin, and pale. Exceptionally ulcers and ecchymoses are observed in the cecum, but generally there are no lesions of the intestinal tract.

The most striking and constant lesions, those most

characteristic of the affection, are in the bile and in the liver; in some cases the quantity of bile may not exceed the normal, but in others the gall-bladder may be abnormally distended with bile. The bile is nearly colorless or has a pale yellowish or brownish tint, with little or no greenish color. Its consistence is much less viscid than normal, being often thin and watery. It usually contains small, opaque, yellowish particles or clumps which can be seen floating in it, even through the walls of the gall-bladder. These clumps consist microscopically of bile-stained, apparently necrotic, epithelial cells; leukocytes in small numbers; amorphous masses of bile-pigment, and bacteria often in zoöglea-like clumps. Similar material is found in the larger bile-ducts.

The liver frequently contains opaque, whitish or yellowish-white spots and streaks of irregular size and shape, which give a peculiar mottling to the organ when present in large number. These areas may be numerous, or only one or two may be found. In size they range from minute points to areas of from 2 to 3 cm. in extent. By microscopic examination they are found to represent localities where the liver-cells have undergone necrosis accompanied by emigration of leukocytes, and the cells about them are in a condition of fatty degeneration. In sections of the liver masses of the bacilli may be discovered in and about the necrotic foci just described.

At these autopsies the colon bacillus is not found generally distributed through the body, but is only to be detected in the bile, liver, and occasionally in the spleen.¹

¹ Consult paper by Blachstein on this subject, *Johns Hopkins Hospital Bulletin*, 1891, ii, 96.

BACILLUS PARATYPHOSUS.

During recent years careful bacteriological examination of cases of continued fever, the blood from which had no agglutinating action upon typhoid bacillus, has revealed a group of bacilli which differ from bacillus typhosus in certain important particulars. These bacteria possess characters which are intermediate between those of bacillus typhosus and bacillus coli, some resembling more closely the former, others the latter, and for these reasons they have sometimes been denominated the intermediate, "near" or "para" group. Some of the organisms isolated from such cases of continued fever resemble very closely bacillus enteriditis, which Gaertner found in cases of meat poisoning.

The general opinion is that these organisms produce a form of infection sometimes resembling in many of its clinical characters that produced by bacillus typhosus. The infection, however, is usually of a milder type and only a comparatively small number of cases have terminated fatally, so that the pathology of the disease is not well known. Moreover, the biological characters of the different organisms isolated from cases of paratyphoid fever, as the condition is called, show such wide variations that it is probable the pathology of different cases also varies with the particular type of organism causing the infection.

Buxton¹ was one of the first to make a careful comparative study of the morphology and biology of this group of organisms. He classifies the intermediary group of organisms in the following manner:

"Paracolons: those which do not cause typhoidal symp-

¹ Journal of Medical Research, viii, 201.

toms in man. A group containing numerous different members, but culturally alike.

“Paratyphoids: those which cause typhoidal symptoms.

“(a) A distinct species culturally unlike the paracolons.

“(b) A distinct species culturally resembling the paracolons.”

Buxton and others state that some of those producing typhoidal symptoms cannot be distinguished culturally from some members of the paracolon group. All the organisms of this intermediate group have the morphological characters of the colon-typhoid group of organisms, and they cannot, therefore, be distinguished from one another by the form or size.

The biological differences on agar-agar, blood serum, gelatin, and bouillon, between the members of the intermediate group, and between *bacillus typhosus* and *bacillus coli* are too insignificant and uncertain to be of any assistance in a differentiation between members of the group. In litmus milk certain well-marked differences between different members of the group are noticed. None of the organisms of the intermediate group produce coagulation. Some produce a slight initial acidity, which is later followed by an alkaline reaction. Still other members of the group produce an acidity amounting to 1 per cent.

Buxton states that the intermediates can be distinguished from *bacillus typhosus* by their power of fermenting the disaccharid maltose and all the monosaccharids with gas formation. On the other hand they can be distinguished from *bacillus coli* by their inability to form acid and gas in *lactose* media.

The agglutination reaction of members of the intermediate group with the serum of an animal immunized with one of

the organisms varies with the different organisms. The more closely a member of the group resembles culturally the organism employed in immunizing the animal the more readily is it agglutinated. In attempts to diagnose paratyphoid infection it is well to bear this fact in mind and make agglutination tests upon different members of the group with the blood of the patient.

CHAPTER XXV.

The Group of Bacilli Found in Cases of Epidemic, Endemic, and Sporadic Dysentery—The Morphological, Biological, and Pathogenic Characters of the Several Members of the Group—The Differentiation of the Different Types of Bacilli.

BACILLUS DYSENTERIÆ.

THE investigations of epidemic dysentery by Shiga, Flexner, Kruse, Vedder, Duval, Basset, Park, and many others, have demonstrated that this disease is caused by an organism that varies somewhat in its characters as encountered in different cases. So far at least four types of organisms have been found that differ in minor particulars, though not sufficiently to warrant their designation as distinct species. The type of organism first encountered by Shiga, in Japan, is the one that is probably very widely distributed, because it has been found in practically every place where search has been made for it. The type of organism encountered by Flexner in the Philippine Islands, and believed by him to differ from the Shiga type, has also been found very generally in the United States, especially in dysentery occurring in infants. The type of organism isolated by Hiss and Russell, and later by Park and his associates, has most of the characteristics of the Flexner type of organism, though the agglutination reaction shows that it is not identical with it.

At first the German investigators were inclined to regard the Flexner type of organism as having no causative relation

whatever to dysentery, but later detailed studies all strengthen the assumption that the Shiga type of the organism is not the only one concerned in causing epidemic dysentery. In a number of cases of dysentery two, and at times three, types of bacillus dysenteriae have been encountered. Thus far it has been impossible to differentiate clinically between the infections produced by the one or the other type, both severe and mild cases being caused by each.

The Shiga Type of Organism.—The evidence presented by Shiga, who discovered this organism in 1898, in Japan, and the subsequent observations of Flexner upon dysentery in the Philippine Islands, leaves little room for doubt that, in so far as acute epidemic dysentery is concerned, the organism under consideration may reasonably be regarded as the causative factor. By both Shiga and Flexner the organism was almost uniformly encountered in the intestinal contents, the intestinal walls, and the mesenteric glands during the acute stages of the disease. Later it was frequently missed, and this became more common as the malady progressed to chronicity or recovery.

It is a bacillus of medium size, with rounded ends. In general its morphology may properly be likened to that of either the typhoid or colon bacillus.

It is motile and does not form spores.

It can be stained with any of the ordinary aniline dyes. It is decolorized by the method of Gram. It may be cultivated on all the ordinary media. It grows at room-temperature, but better at the temperature of the body. It does not liquefy gelatin.

The colonies upon agar-agar present nothing characteristic; those on gelatin are at first—*i. e.*, just after isolation from the body—like those of bacillus typhosus; later on,

after the organism has been kept under conditions of continuous saprophytic growth, the colonies may be thicker, denser, moister, and less translucent, but always suggesting the peculiar, leaf-like contour characteristic of the colonies of the colon-typhoid group under similar conditions. In gelatin stab-cultures there is growth along the track made by the needle, and little tendency to lateral development over the surface.

On potato, its growth may be so limited as to be scarcely visible, or it may appear as a moderately voluminous grayish-brown or light-brown layer along the track made by the needle, and spreading laterally beyond this. Between these extremes all gradations may be seen according to the suitability of the potato used.

In bouillon it causes uniform clouding and a more or less dense sediment. It does not form a pellicle.

Growth on blood-serum is not accompanied by liquefaction (digestion).

Glycerin-agar-agar appears less suited to its growth than plain nutrient agar-agar.

It does not ferment either glucose, saccharose, or lactose, with liberation of gas; although in glucose media there is a slight increase of acidity.

When grown in litmus-milk, the latter, after twenty-four to seventy-two hours at body-temperature, becomes a pale lilac. Later on—*i. e.*, after six to eight days—there is a development of alkali, and the lilac tint gives way to a deep, distinct blue color. Coagulation is never observed.

It is either incapable of producing indol, or has this faculty developed to so limited a degree as to make the matter doubtful.

When mixed with blood-serum of individuals suffering

from this form of dysentery a positive agglutination reaction is often obtained.

It is pathogenic by both subcutaneous and intraperitoneal inoculation for the ordinary laboratory test-animals—*i. e.*, mice, guinea-pigs, and rabbits.

When injection is made beneath the skin, death results in from two to four days, according to the dose and virulence of the culture used.

The most striking lesion is that observed at and about the site of inoculation. This consists of edema, hemorrhagic exudation, and in delayed cases, more or less of pus formation. The subcutaneous lymph-glands are often enlarged and reddened, and a serous exudation is frequently encountered in the great serous cavities. Of the animals mentioned, the rabbit is most apt to survive the subcutaneous inoculation.

When injected into the peritoneal cavity, death takes place in from a few hours to five or six days, according to dose and virulence of the culture used.

At autopsy the superficial lymph-glands are enlarged and reddened; the peritoneum contains more or less of turbid fluid and small masses of leukocytes; the pleural and pericardial cavities may contain clear fluid; the spleen is swollen; the adrenals and kidneys are congested; there may be a grayish exudate over the liver, spleen, and intestines, the bloodvessels are injected; the small intestine may be filled with semifluid or fluid matter; there may be ecchymosis in the intestinal mucosa, and Peyer's patches may be enlarged and reddened.

The distribution of the bacilli varies: sometimes there is a general invasion of the body by the bacilli; at others they are only to be found at the local site of inoculation.

Sometimes they can be detected in the intestinal contents after both subcutaneous and intraperitoneal inoculation; at other times they cannot.

If the stomach contents be neutralized and large doses of the bacilli be administered *per os*, death may occur. Under these conditions the small intestine is hyperemic and contains blood-stained mucoid matter, from which the bacilli may usually be cultivated.

If cultures be fed to cats after administration of croton oil, a fatal diarrhea may ensue. The mucous membrane of the large intestine is injected, its surface covered with mucous, and its contents mucoid. From the latter the bacilli may be recovered in culture.

A fatal diarrhea may follow the simple feeding of cultures to dogs. This occurs in somewhat less than six days. The condition of the contents and walls of the large intestine is essentially similar to that seen in the cat.

In view of the fact that marked evidences of intoxication may follow upon the injection of suspensions of dead cultures of this organism (solid cultures killed by exposure to 60° C.), it is probable that the pathogenicity of this organism is referable to its endotoxin, rather than to a soluble intoxicant secreted or manufactured as a by product in the course of growth.

The Hiss-Russell Type of Organism.—In the detailed study of dysentery and summer diarrhea in infants, a type of bacillus dysenteriae has been encountered which has the property of fermenting mannite as well as dextrose. The Shiga type ferments dextrose, but none of the other carbohydrates.

The Strong Type of Organism.—This type of organism has many of the characters of the Harris type, though it ferments only mannito-dextrose, and saccharose.

The Harris Type of Organism.—This type of bacillus dysenteriae was first encountered by Strong while working in the Philippine Islands. It has since been encountered quite frequently in the United States, especially in the summer diarrheas in infants. This organism ferments mannite as well as dextrose, maltose, saccharose, and dextrin.

It is only by careful observations of the reactions with the different carbohydrates that it is possible by culture methods to differentiate between these different strains of bacillus dysenteriae, as has been shown by Hiss¹ and by others.

The Agglutinability of Bacillus Dysenteriae.—The influence of agglutinins in dysentery immune serum has also served to differentiate between different types of bacillus dysenteriae. Normal serums, especially those of bovines and of goats, also yield very instructive results. Variations in the agglutinability of the several types of bacillus dysenteriae, especially in normal serums, were first pointed out by Bergey,² and have since been noticed by other investigators (see especially Park and Hiss, *loc. cit.*).

The different types of bacillus dysenteriae can easily be distinguished by their relative agglutinability, but in order to do so animals must be rendered immune from each variety and the serum of such animals employed as specific reagents. When this is done it will be found that the serum of an animal immunized with the Shiga type of organism will agglutinate that type of organism in high dilutions, say 1 : 5000, while the Harris type of organism will only be agglutinated in dilutions of 1 : 200, and the Hiss-Russell

¹ Journal of Medical Research, December, 1904, viii.

² Ibid., 1903, v, 21.

type of organism in dilutions of 1 : 50. On the other hand, the serum of an animal immunized with the Flexner type of organism will agglutinate that type of organism in high dilutions, say 1 : 10,000, while the other two types of the organism will be agglutinated only in dilutions of 1 : 100. The serum of an animal immunized with the Hiss-Russell type of organism will agglutinate that type of organism in dilutions, say of 1 : 1000, while the Harris type is agglutinated only in dilutions of 1 : 100, and the Shiga type in dilutions of 1 : 20.

Protective Inoculation.—By the repeated inoculation of animals with cultures of this organism, killed either by heat or by chemicals, it has been found possible to protect them against otherwise fatal doses of the living virulent organism. When treated in this way, the goat supplies a serum that exhibits not only an agglutinating power over the living bacilli, but possesses both protective and curative properties when injected into other susceptible animals.

During 1898–1899 Shiga¹ employed a protective serum, made after the foregoing principles, in the treatment of dysentery in human beings. During the period mentioned he treated 266 cases, and had a death-rate of 9.6 per cent.; while for 1736 cases occurring at the same time and in the same locality, but not so treated, there was a death rate of 34.7 per cent.²

Holt³ summarizes the results obtained in the treatment

¹ See *The Epidemic Dysentery of the Past Twenty Years in Japan*, by Stuart Eldridge, M.D., U. S. Marine-Hospital Service, *Public Health Reports*, 1900, xv, No. 1, 1–11.

² The foregoing sketch is compiled from:

Shiga, Ueber den Dysenterie-bacillus (*Bacillus dysenteriae*), *Centralblatt für Bakteriologie und Parasitenkunde*, 1898, Abt. i, Bd. xxiv, Nos. 22, 23, 24.

Flexner, On the Etiology of Tropical Dysentery, *Philadelphia Medical Journal*, September 1, 1900.

³ *Studies from the Rockefeller Institute for Medical Research*, 1904, vol. ii.

of 87 cases with dysentery immune serum. Decided improvement was noted in only 12 of the patients. These were principally hospital cases, and hence rather grave forms of the disease. Another factor which probably operated against the favorable influence of the serum is the fact that the serum treatment was generally preceded by a careful bacteriological analysis of the stools in order to establish a positive diagnosis, requiring two or three days so that the serum treatment was instituted late in the course of the disease.

Holt points out that the conditions necessary to obtain success in the serum treatment of cases of dysentery are: First, the early use of the serum, before serious lesions have developed or before the patient's general condition has been too profoundly impaired; second, the serum must be administered in repeated doses, one or two doses a day, and continued for several days in severe cases.

CHAPTER XXVI.

The Spirillum (Comma Bacillus) of Asiatic Cholera—Its Morphological and Cultural Peculiarities—Pathogenic Properties—The Bacteriological Diagnosis of Asiatic Cholera—Microspira Metchnikovi—Microspira ("Vibrio") Schuylkilliensis—Its Morphological, Cultural, and Pathogenic Characters.

THE CHOLERA GROUP OF ORGANISMS.

At the conference held in Berlin in 1884 for the purpose of discussing Asiatic cholera from the sanitary aspect, it was announced by Koch¹ that he had discovered in the intestinal evacuations of individuals suffering from Asiatic cholera a micro-organism that he believed to be the cause of the malady. The importance of this statement naturally attracted widespread attention to the subject, and as one of the consequences there existed, for a short time following, some skepticism as to the accuracy of Koch's claim. These doubts arose as a result of a series of contributions from other observers, who endeavored to prove that the organism found by Koch in cholera evacuations was common to other localities, and was not a specific accompaniment of this disease. It was not very long, however, before it was evident that these objections were based upon untrustworthy observations, and that by reliable methods of investigation the organism to which he had called attention could be easily differentiated from each of those with which it was claimed to be identical.

¹ Verhandlungen der Conferenz zur Erörterung der Cholerafrage, 1884, Berlin.

This organism, commonly known both as the spirillum of Asiatic cholera, and, because of its morphology, as Koch's "comma bacillus," is identified by the following peculiarities:

MICROSPIRA COMMA (KOCH), SCHRÖTER, 1886.

SYNONYMS. Comma-bacillus, Koch, 1884; Spirillum cholerae Asiatica, Flügge, 1886.

Morphology.—It is a slightly curved rod, ranging from about 0.8 to 2μ in length and from 0.3 to 0.4μ in thickness—that is to say, it is usually from about one-half to two-thirds the length of the tubercle bacillus, but is thicker and plumper. Its curve is frequently not more marked than that of a comma, and, indeed, it is often almost straight; at times, though, the curve is much more pronounced, and may even describe a semicircle. Occasionally the curve may be double, one comma joining another, with their convexities pointing in opposite directions, so that a figure similar to the letter S is produced. In cultures long spiral or undulating threads may often be seen. From these appearances this organism cannot be considered as a bacillus, but rather as an intermediate type between the bacilli and the spirilla. Koch thinks it not improbable that the short comma forms represent segments of a true spirillum, the normal form of the organism. (Fig. 86.)

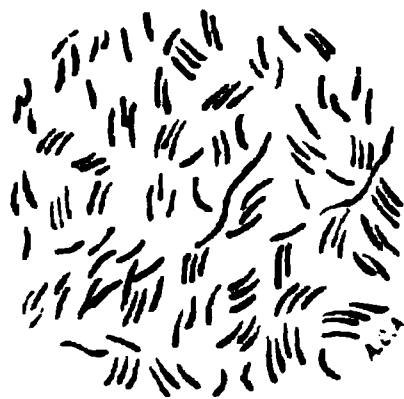
It does not form spores, and we have no reliable evidence that it possesses the property of entering, at any time, a stage in which its powers of resistance to detrimental agencies are increased.

It is a flagellated organism, but has only a single flagellum attached to one of its ends.

It is actively motile, especially in the comma stage though the long spiral forms also possess this property.

GROUPING.—As found in the slimy flakes in the intestinal discharges from cholera patients, Koch likens its mode

FIG. 88



Microspira comma. Impression cover-slip from a colony thirty-four hours old.

of grouping to that seen in a school of small fish when swimming up stream—*i. e.*, they all point in nearly the

FIG. 89



Involution-forms of *microspira comma*, as seen in old cultures.

same direction, and lie in irregularly parallel, linear groups that are formed by one comma being behind the other without being attached to it.

On cover-slip preparations made from cultures in the

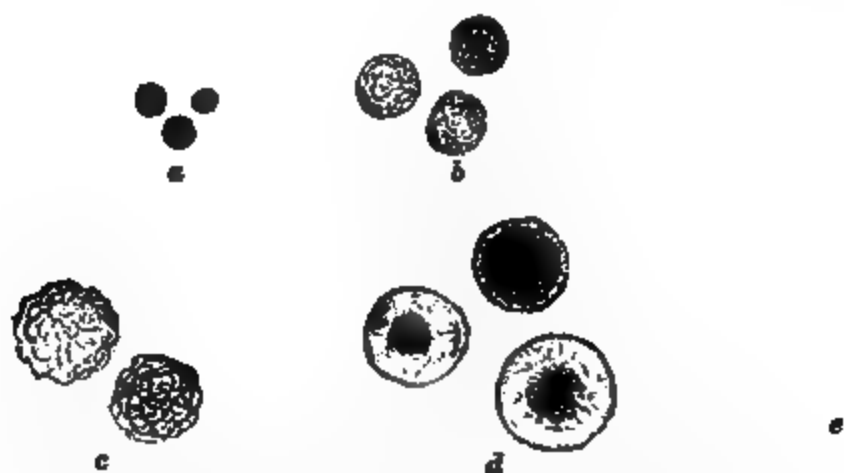
ordinary way there is nothing characteristic about the grouping; but in impression cover-slips made from young cultures the short commas will nearly always be seen in small groups of three or four, lying together in such a way as to have their long axes nearly parallel to one another. (See Fig. 88.)

In old cultures in which development has ceased it undergoes degenerative changes, and the characteristic comma and spiral shapes may entirely disappear, their place being taken by irregular involution-forms that present every variety of outline. (See Fig. 89.) In this stage they take on the stain very feebly, and often not at all.

Cultural Peculiarities.—On plates of nutrient gelatin that have been prepared from a pure culture of this organism and kept at a temperature of from 20° to 22° C., development can often be observed after as short a period as twelve hours, but frequently not before sixteen to eighteen hours. This is especially true of the first or "original" plate, containing the largest number of colonies. At this time the plate will present to the naked eye an appearance that has been likened to a ground-glass surface, or to a surface that has been stippled with a finely pointed needle, or one upon which very fine dust has been sprinkled. This appearance is due to the presence of minute colonies closely packed together upon the surface of the gelatin. In the depth of the gelatin can also be seen closely packed, small points, likewise representing growing colonies. As growth progresses liquefaction occurs around the superficial colonies, and in consequence this plate is usually entirely liquid after from twenty-four to thirty hours; the developmental phases through which the colonies pass cannot, therefore, be studied upon it.

On plates 2 and 3, where the colonies are more widely separated, they can be seen after twenty-four to thirty hours as small, round or oval, white or cream-white points, and when located superficially a narrow transparent zone of liquefaction can be detected around them. As growth continues this liquefaction extends downward rather than laterally, and the colony ultimately assumes the appearance of a dense, white mass lying at the bottom of a sharply-cut

FIG. 90



Development phases of colonies of *microspira comma* at 20° to 22° C. on gelatin. \times about 75 diameters. *a*, after sixteen to eighteen hours; *b*, after twenty-four to twenty-six hours; *c*, after thirty-eight to forty hours; *d*, after forty-eight to fifty hours; *e*, after sixty-four to seventy hours.

pit or funnel containing transparent fluid. This liquefaction is never very widespread nor rapid, and rarely extends more than one millimeter beyond the colony proper. On plates containing few colonies there is little or no tendency for them to become confluent, and they rarely exceed 2 to 3 mm. in diameter.

When examined under a low magnifying lens the very young colonies (sixteen to eighteen hours old) appear as pale, translucent, granular globules of a very delicate

greenish or yellowish-green color, sharply outlined, and not perfectly round. (See *a*, Fig. 90.) As growth progresses this homogeneous granular appearance is replaced by an irregular lobulation, and ultimately the sharply-cut margin of the colony becomes dentated or scalloped. (See *b* and *c*, Fig. 90.) After forty-eight hours (and frequently sooner) liquefaction of the gelatin has taken place to such an extent that the appearance of the colony is entirely altered. Under a magnifying glass the colony proper is now seen to be ragged about its edges, while here and there shreds of the colony can be detected scattered through the liquid into which it is sinking. These shreds evidently represent portions of the colony that became detached from its margin as it gradually sank into the liquefied area.

At *d*, in Fig. 90, is seen a representation of the several appearances afforded by the colonies at this stage. At the end of the second, or during the early part of the third day, the sinking of the colonies into the liquefied pits resulting from their growth is about complete, and under a low-power lens they now appear as dense, granular masses, surrounded by an area of liquefaction through which can be seen granular prolongations of the colony, usually extending irregularly between the periphery and the central mass. (See *e*, Fig. 90.) If the periphery be examined, it will be seen to be fringed with delicate, cilia-like lines that radiate from it in much the same way that cilia radiate from the ends of the columnar epithelial cells lining the air-passages.

These are the more marked phases through which the colonies of this organism pass in their development on gelatin plates. In some cultures the various phases here given pass in succession more quickly, while in cultures from other sources they may be somewhat retarded.

On plates of nutrient agar-agar the appearance of the colonies is not characteristic. They appear as round or oval patches of growth that are moist and moderately transparent. The colonies on this medium at 37° C. naturally grow to a larger size than do those upon gelatin at 22° C.

FIG. 91

a b c d

Stab-culture of *microspira comma* in gelatin, at 18° to 20° C. a, after twenty-four hours; b, after forty-eight hours; c, after seventy-two hours; d, after ninety-six hours.

In stab-cultures in gelatin there appears at the top of of the needle-track after thirty-six to forty-eight hours at 22° C. a small, funnel-shaped depression. As the growth progresses liquefaction occurs about this point. In the centre of the depression can be distinguished a small, dense,

whitish clump, the colony itself. As growth continues the depression increases in extent and ultimately assumes an appearance that consists in the apparent sinking of the liquefied portion in such a way as to leave a perceptible air-space between the top of the liquid and the surface of the solid gelatin. The growth now appears to be capped by a small air-bubble. The impression given by it at this stage is not only that there has been a liquefaction, but also a coincident evaporation of the fluid from the liquefied area and a constriction of the superficial opening of the funnel. (See *a*, *b*, *c*, and *d*, Fig. 91.) Liquefaction is not especially active along the deeper portions of the track made by the needle, though in stab-cultures in gelatin the liquefaction is much more extensive than that usually seen around colonies on plates. It spreads laterally at the upper portion, and after about a week a large part of the gelatin in the tube may have become fluid, and the growth will have lost its characteristic appearance.

Stab- and smear-cultures on agar-agar present nothing characteristic.

Its growth in bouillon is luxuriant, causing a diffuse clouding and the ultimate production of a delicate film upon the surface.

In sterilized milk of a neutral or amphoteric reaction at a temperature of 36° to 38° C. it develops actively, and gradually produces an acid reaction, with coagulation of the casein. It retains its vitality under these conditions for about three weeks or more. The blue color of milk to which neutral litmus tincture has been added is changed to pink after thirty-six or forty-eight hours at body-temperature.

Its growth in peptone solution, either that of Dunham (see Special Media) or the one preferred by Koch, viz.,

2 parts of Witte's peptone, 1 part of sodium chloride, and 100 parts of distilled water, is accompanied by the production of both indol and nitrites, so that after eight to twelve hours in the incubator at 37° C. the rose color characteristic of indol appears upon the addition of sulphuric acid alone. (See Indol Reaction.)

(What does the presence of nitrites in these cultures signify?)

In peptone solution to which rosolic acid has been added the red color is very much intensified after four or five days at 37° C.

Its growth on potato of slightly acid reaction is seen after three or four days at 37° C. as a dull, whitish, non-glistening patch at and about the site of inoculation. It is not elevated above the surface of the potato, and can only be distinctly seen when held to the light in a particular position. Growth on acid potato occurs, however, only at or near the body-temperature, owing probably to the acid reaction, which is sufficient to prevent development at a lower temperature, but does not have this effect when the temperature is more favorable. On solidified blood-serum growth is usually said to be accompanied by slow liquefaction. I have not succeeded in obtaining this result on Löffler's serum, nor have I detected anything characteristic about its growth on this medium.

The temperature most favorable for its growth is between 35° and 38° C. It grows, but more slowly, at 17° C. Below 16° C. no growth is visible.

It is not destroyed by freezing. When exposed to 65° C. its vitality is destroyed in five minutes.

It is strictly aërobic, its development ceasing if the supply of oxygen be cut off.

It does not grow in an atmosphere of carbonic acid, but is not killed by a temporary exposure to this gas. It does not grow in acid media, but flourishes best in media of neutral or slightly alkaline reaction. It is so sensitive to the action of acids that at 22° C. its development is arrested when an acid reaction equivalent to 0.066 to 0.08 per cent. of hydrochloric or nitric acid is present. (Kitasato.)

Under artificial cultivation the maximum development of this organism is reached in a comparatively short time; after this it remains quiescent for a period, and finally degeneration or involution begins. When in this state they take up coloring-reagents very faintly or not at all, and may lose entirely their characteristic shape. (See Fig. 93.)

When present with other bacteria, under conditions favorable to growth, the comma bacillus at first grows much more rapidly than do the others; in twenty-four hours it will often so outnumber the other organisms present that microscopic examination might lead one to regard the material under consideration as a pure culture of this organism. Its conspicuous development under these circumstances does not, however, last longer than two or three days; degeneration and death begin, and the other organisms gain the ascendancy. This fact has been taken advantage of in the bacteriological diagnosis of cholera.

In connection with his experiments upon the poison produced by the cholera organism Pfeiffer¹ states that in very young cultures, grown under access to oxygen, there is present a body that possesses intensely toxic properties. This primary cholera-poison stands in very close relation to the material composing the bodies of the bacteria them-

¹Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xi, S. 393.

selves, and is probably an integral constituent of them, for the vitality of the cholera spirilla can be destroyed by means of chloroform or thymol, or by drying, without apparently any alteration of this poisonous body. Absolute alcohol, concentrated solutions of neutral salts, and a temperature of 100° C., decompose this substance, leaving intact secondary poisons which possess a similar physiological activity, but only when given in from ten to twenty times the dose necessary to produce the same effects with the primary poison.

Experiments upon Animals.—As a result of experiments for the purpose of determining if the disease can be produced in any of the lower animals it has been found that white mice, monkeys, cats, dogs, poultry, and many other animals are not susceptible to infection by the methods usually employed in inoculation experiments. When animals are fed on pure cultures of the comma bacillus no effect is produced, and the organisms cannot be obtained from the stomach or intestines. They are destroyed in the stomach, and do not reach the intestines; they are not demonstrable in the feces of these animals. Intravascular injections of a pure culture into rabbits are followed by an illness, from which the animals usually recover in from two to three days; intraperitoneal injections into white mice are, as a rule, followed by death in from twenty-four to forty-eight hours, the conditions in both instances most probably resulting from the toxic activities of the specific poisons contained in the cultures used.

None of the lower animals suffer spontaneously from Asiatic cholera.

The failure to induce cholera in animals by feeding or by injection of cultures into the stomach, was shown by

Nicati and Rietsch¹ to be due to the destructive action of the acid gastric juice on the organisms. They showed that if cultures of this organism were introduced into the alimentary tract of certain animals in such a manner that they would not be subjected to the influence of the gastric juice, a pathological condition closely simulating cholera as it occurs in man could be produced.' For this purpose the common bile-duct was ligated, after which the cultures were injected directly into the duodenum. Such interference with the flow of bile lessens intestinal peristalsis, and thus permits development of the organisms at the point at which they are deposited—that is, the portion of the intestine having an alkaline reaction and beyond the influence of the acid stomach-juice.

By this method Nicati and Rietsch, Van Ermengem,² Koch,³ and others were enabled to produce in the animals upon which they operated a condition that was, if not identical, at all events very similar pathologically to that seen in the intestines of subjects dead of the disease.

At a subsequent conference held in Berlin in 1885 Koch⁴ described the following method, by means of which he had been able to obtain a much greater degree of constancy in his efforts to produce cholera in lower animals: bearing in mind the point made by Nicati and Rietsch as to the effect produced by the acid reaction of the gastric juice, this reaction was first to be neutralized by injecting through a soft catheter passed down the esophagus into the stomach 5 c.c. of a 5 per cent. solution of sodium carbonate. Ten

¹ Archiv de Phys. norm. et path., 1885, t. vi. 3e sér. Comptes rendus, xcix, p. 928; Revue de Hygiène, 1885; Revue de Médecine, 1885, v.

² Recherches sur le Microbe du Choléra Asiatique, Paris-Bruxelles, 1885; Bull. de l'Acad. roy. de Méd. de Belgique, xviii, 3e sér.

³ Loc. cit.

⁴ Loc. cit.

or fifteen minutes later this was to be followed by the injection into the stomach (also through a soft catheter) of 10 c.c. of a bouillon culture of *microspira comma*. For the purpose of arresting peristalsis and permitting the bacteria to remain in the stomach and upper part of the duodenum for as long a time as possible, the animal was to receive, immediately following the injection of the culture, an intraperitoneal injection, by means of a hypodermic syringe, of 1 c.c. of tincture of opium for each 200 grams of its body-weight. Shortly after this last injection deep narcosis sets in and lasts from a half to one hour, after which the animal is as lively as ever. Of 35 guinea-pigs inoculated in this way by Koch, 30 died of an affection that was, in general, very similar to Asiatic cholera as seen in man.

The condition of those animals before death is described as follows: twenty-four hours after the operation the animal appears unwell; there is loss of appetite, and the animal remains quiet in its cage. On the following day a paralytic condition of the hind extremities appears, which, as the day wears on, becomes more pronounced; the animal lies quite flat upon its abdomen or on its side, with legs extended; respiration is weak and prolonged, and the pulsations of the heart are hardly perceptible; the head and extremities are cold, and the body-temperature is frequently subnormal. The animal usually dies after remaining in this condition for a few hours.

At autopsy the small intestine is found deeply injected and filled with flocculent, colorless fluid. The stomach and intestines do not contain solid masses, but fluid; when diarrhea does not occur, firm scybala may be detected in the rectum. Both by microscopic examination and by cul-

ture methods the organisms are found present in the small intestine in practically pure culture.

More recently Pfeiffer¹ has determined that essentially similar constitutional effects may be produced in guinea-pigs by the intraperitoneal injection of relatively large numbers of this organism. His plan is to scrape from the surface of a fresh culture on agar-agar as much of the growth as can be held upon a medium size wire loop. This is then finely divided in 1 c.c. of bouillon, and by means of a hypodermic syringe is injected directly into the peritoneal cavity. When virulent cultures have been used this operation is quickly followed by a fall in the temperature of the animal that is gradual and continuous until death ensues, which usually occurs in from eighteen to twenty-four hours after the operation, though exceptionally the animal recovers, even after having exhibited marked symptoms of profound toxemia.

Continuing his studies upon this disease, Pfeiffer² has demonstrated that it is possible to render an animal immune from the poisonous properties of this organism by repeated injections of non-fatal doses of dead cultures (cultures that have been killed by the vapor of chloroform or by heat). He also demonstrated that animals so immunized possess a specific germicidal action toward *microspira comma*—*i. e.*, if into the peritoneal cavity of an animal immunized from Asiatic cholera living organisms be introduced they will all be destroyed (disintegrated) within a relatively short time. Furthermore, if the serum of an animal immunized from cholera be injected into the peritoneal cavity of another animal of the same species, but not so protected, and imme-

¹ Zeitschrift für Hygiene und Infektionskrankheiten, Bd. xi and xiv.

² Ibid., 1894, Bd. xvii, S. 355; 1894, Bd. xviii, S. 1; 1895, Bd. xx, S. 197.

diately afterward living cholera spirilla be introduced, a similar disintegration and destruction of the bacteria will also result. He shows that a more or less definite relation exists between the amount of serum and the number of organisms introduced. Such a destruction of *microspira comma* by the serum of an immunized animal does not occur outside the animal body—that is, it cannot be demonstrated in a test-tube, unless, as Bordet has demonstrated, it be perfectly fresh from the animal body, or, as Metchnikoff has shown, there be added to it a small quantity of fresh serum from a normal guinea-pig. The specificity of this reaction is suggested by Pfeiffer as a means of differentiating the cholera spirillum from other suspicious species, for no such bacteriolytic action is observed if other bacteria be introduced into the peritoneal cavity of animals immunized from Asiatic cholera.

Pfeiffer has further demonstrated that the serum of animals artificially immunized from Asiatic cholera has an agglutinating effect upon fluid cultures of *microspira comma* similar to that seen when typhoid bacilli are mixed with serum from typhoid cases, or from animals artificially immunized from typhoid infection or intoxication. (See Agglutinin.)

General Considerations.—In all cases of Asiatic cholera, and *only* in this disease, the organism just described can be detected in the intestinal evacuations. The more acute the case and the more promptly the examination is made after the evacuations have passed from the patient, the less difficulty will be experienced in detecting the organism.

In some cases the organism can be detected in the vomited matters, though by no means so constantly as in the intestinal contents.

As a rule, bacteriological examination fails to reveal the presence of the organisms in the blood and internal organs in this disease, though exceptions have been noted.

Microspira comma is a facultative saprophyte; that is to say, it apparently finds in certain parts of the world, particularly in those countries in which Asiatic cholera is endemic, conditions that are not entirely unfavorable to its development outside of the body. This was found to be the case not only by Koch, who detected the presence of the organism in water-tanks in India, but by many other observers who have succeeded in demonstrating its growth under conditions not embraced in the ordinary methods employed for the cultivation of bacteria.¹

The results of experiments having for their object the determination of the length of time during which the organism may retain its vitality in water are conspicuous for their irregularity.

Koch states that in ordinary spring-water or well-water the organisms retained their vitality for thirty days, whereas in the sewage of Berlin they died after six or seven days; but if this latter were mixed with fecal matters, the organisms retained their vitality for but twenty-seven hours; and in the undiluted contents of cesspools it was impossible to demonstrate them after twelve hours. In the experiments of Nicati and Rietsch they retained their vitality in Marseilles sewage for thirty-eight days; in sea-water, sixty-four days; in harbor-water, eighty-one days; and in bilge-water, thirty-two days.

In one test with the water-supply of Berlin the organism

¹ Obviously all pathogenic bacteria that have been isolated under artificial methods of cultivation are *facultative* saprophytes. Were they *obligate* parasites, their cultivation upon dead materials would be impossible.

retained its vitality for 267 days, and in another for 382 days, notwithstanding the fact that many other organisms were present at the same time. There is no ready explanation for these variations, for they depend apparently upon a number of factors which may act singly or together. For example, in general it may be said that the higher the temperature of the water in which these organisms are present, up to 20° C., the longer do they retain their vitality; the purer the water—that is, the poorer in organic matters—the more quickly do the organisms die, whereas the richer it is in organic matter the longer do they retain their vitality.

The effect of light upon growing bacteria must not be lost sight of, for it has been shown that a surprisingly large number of these organisms are robbed of their vitality by a relatively short exposure to the direct rays of the sun; and it is therefore not unlikely that the non-observance of this fact may be, in part at least, accountable for some of the discrepancies that appear in the results of these experiments.

In his studies upon the behavior of pathogenic and other micro-organisms in the soil Carl Fränkel¹ found that *microspira comma* was not markedly susceptible to those deleterious influences that cause the death of a number of other pathogenic organisms. At a depth of one and a half meters vitality was not destroyed, and there was a regular development in cultures so placed.

As a result of experiments performed in the Imperial Health Bureau at Berlin, it was found that the bodies of guinea-pigs that had died of cholera induced by Koch's method of inoculation contained no living cholera spirilla when exhumed after having been buried for nineteen days in wooden boxes, or for twelve days in zinc boxes. In a

¹ *Zeitschrift für Hygiene*, Bd. ii, S. 521.

few that had been buried in moist earth, without having been encased in boxes, when exhumed after two or three months, the results of examinations for cholera spirilla were likewise negative.

Esmarch¹ found that when the cadaver of a guinea-pig dead after the introduction of cholera organisms into the stomach was immersed in water until decomposition was far advanced, it was impossible to find any living microspira comma by the ordinary plate methods. Several experiments resulted in their disappearance in five days. In one experiment, in which decomposition was allowed to go on without the animal being immersed in water, none could be detected after the fifth day.

Kitasato² found that when mixed with the normal intestinal evacuations of human beings it lost its vitality in from a day and a half to three days. If the evacuations were sterilized before the cultures were mixed with them it retained its vitality from twenty to twenty-five days.

Hesse³ and Celli⁴ demonstrated that many substances commonly employed as food serve as favorable materials for the development of the cholera organisms.

Kitasato⁵ found that at 36° C. microspira comma developed very rapidly in milk during the first three or four hours, and outnumbered the other organisms commonly present. It then diminished in number from hour to hour as the acidity of the milk increased, until finally its vitality was lost; at the same time the common saprophytic bacteria increased in number. Relatively the same process

¹ v. Esmarch, Zeitschrift für Hygiene, Bd. vii, S. 1.

² Zeitschrift für Hygiene, Bd. v, S. 487.

³ Ibid., S. 527.

⁴ Bolletino della R. Acad. Med. di. Roma, 1888.

⁵ Zeitschrift für Hygiene, Bd. v, S. 491.

occurs at a lower temperature, from 22° to 25° C.; but it is slower, the maximum development of the cholera organisms being reached at about the fifteenth hour, after which time they were outnumbered by the ordinary saprophytes present.

From the foregoing it would seem that the vitality of *microspira comma* in milk depends largely upon the reaction; the more quickly the milk becomes sour the more quickly does the organism become inert.

According to Laser,¹ the cholera organism retains its vitality in butter for about seven days; it is therefore possible for the disease to be contracted by the use of butter that has in any way been in contact with cholera material.

When dried *microspira comma* retains its vitality for from about three to twenty-four hours, according to the degree of desiccation. In moist conditions vitality may be retained for many months; though repeated observations lead us to believe that under these circumstances virulence is diminished.

Carbon dioxide, carbon monoxide and nitrous oxide gases kill this germ in from seven to ten days.

From what has been said, we see that the spirillum of Asiatic cholera, while possessing the power of producing in human beings one of the most rapidly fatal diseases with which we are acquainted, is still one of the least resistant of the pathogenic organisms known to us. Under conditions most favorable to its growth its development is self-limited; it is markedly susceptible to acids, alkalies, other chemical disinfectants, and heat; but when partly dried upon clothing, food, or other objects, it may retain its vitality for a relatively long period of time, and it is more than probable

¹ Zeitschrift für Hygiene, Bd. x, S. 513.

that in this way the disease is often disseminated from points in which it is epidemic or endemic into localities that are free from it.

THE DIAGNOSIS OF ASIATIC CHOLERA BY BACTERIOLOGICAL METHODS.

Because of the manifold channels that are open for the ready dissemination of this disease it is of the utmost importance that it should be recognized as quickly as possible, for with every moment of delay opportunities for its spread multiply. It is essential, therefore, when employing bacteriological means for making the diagnosis, to bear in mind those biological and morphological features of the organism that appear most quickly under artificial methods of cultivation, and which, at the same time, may be considered as characteristic of it, viz., its peculiar morphology and grouping; the much greater rapidity of its growth over that of other bacteria with which it may be associated; the characteristic appearance of its colonies on gelatin plates and of its growth in stab-cultures in gelatin; its property of producing indol and coincidently nitrites in from six to eight hours in peptone solution at 37° to 38° C.; and its power of causing the death of guinea-pigs in from sixteen to twenty-four hours when introduced into the peritoneal cavity, death being preceded by symptoms of extreme toxemia, characterized by prostration and gradual and continuous fall in the temperature of the animal's body.

Koch¹ devised a plan of procedure that comprehends the points just enumerated. By its employment the diagnosis can be established in the majority of cases of Asiatic cholera

¹ *Zeitschrift für Hygiene und Infektionskrankheiten*, 1893, Bd. xiv, S. 319.

in from eighteen to twenty-two hours. In general, the steps to be taken and points to be borne in mind are as follows:

1. **Microscopic Examination.**—From one of the small slimy particles seen in the semi-fluid evacuations, obtained as soon as possible after their passage, prepare a cover-slip preparation in the ordinary way and stain it. If, upon microscopic examination, *only* curved rods, or curved rods greatly in excess of all other forms, are present, the diagnosis of Asiatic cholera is *more than likely* correct; and particularly is this true if these organisms are arranged in irregular linear groups with the long axes of all the rods pointing in nearly the same direction.

2. **Plate Cultures.**—From another slimy flake prepare a set of gelatin plates. Keep them at a temperature of from 20° to 22° C., and after sixteen, twenty-two, and thirty-six hours observe the appearance of the colonies. Usually after about twenty-two hours the colonies of this organism can easily be identified by one familiar with them.

3. **Peptone Cultures.**—With another slimy flake start a culture in a tube of peptone solution—either the solution of Dunham or, as Koch proposes, a solution of double the strength of that of Dunham (Witte's peptone is to be used, as it gives the best and most constant results). Keep this at from 37° to 38° C., and at the end of from *six to eight* hours prepare cover-slips from the *upper layers* (without shaking) and examine them microscopically. If comma bacilli were present in the original material, and are capable of multiplication, they will be found in this locality in almost pure culture. After the microscopic examination prepare a second peptone culture from the *upper layers* of the one just examined, also a set of gelatin plates, and with what

remains make the test for indol by the addition of 10 drops of concentrated sulphuric acid for each 10 c.c. of fluid contained in the tube. If comma bacilli are growing in the tube, the rose color characteristic of the presence of indol should appear.

By following this plan "a bacteriologist who is familiar with the morphological and biological peculiarities of this organism should make a more than *probable* diagnosis at once by microscopic examination alone, and a *positive* diagnosis in from twenty to, at most, twenty-four hours after beginning the examination." (Koch.)

Since the publication of the foregoing plan many other methods have been suggested. They all comprehend the "enrichment," by special culture methods, of the number of cholera organisms in the original material without at the same time encouraging the multiplication of the other bacteria present, and the subsequent isolation of the cholera organism by the use of selective plating media. Of these methods, the following gives general satisfaction and can be recommended:

1. Enrichment in the peptone solution exactly as recommended above by Koch if it be intestinal contents that are under consideration; if it be water or sewage, then add to 90 c.c. of the water or sewage in an Erlinger flask 10 c.c. of a 10 per cent. solution. Keep at 37° to 38° C. for about eight hours.

2. Without shaking the tube or flask, now transfer one wire loopful from the *surface* of the mixture of feces, water or sewage and peptone solution, to several tubes containing the Benedict¹ medium:

Water	1000 c.c.
Peptone	10 c.c.
Sodium chloride	5 c.c.

¹ Cent. of Bact., etc., Abt. i, Bd. lxii, S. 536.

Boil and render neutral to phenolphthalein.

Add 1 gram of anhydrous sodium carbonate; boil and filter through double filter paper. Add:

Saccharose	5 grams
Phenolphthalein (sat. sol. in 50 per cent. alcohol)	5 c.c.

Tube and sterilize by steam at 100° C.

The phenolphthalein in this alkaline solution gives to the tubes a bright, rose-red color.

As the vibrios ferment saccharose rapidly, with resultant acid production, the tubes containing them are quickly decolorized. One, therefore, discards all tubes that are not decolorized after eight hours at 37° C. Those that are decolorized may contain cholera vibrios or other closely allied spirilla or any of the group of bacteria having the power to ferment saccharose. The isolation of the cholera spirilla from this possible mixture is now accomplished by differential or selective plating.

3. Of the many differential plating media recommended, that which gives uniformly satisfactory results is the alkaline egg medium recommended by Krumwiede, Pratt and Grund,¹ and slightly modified by Goldberg:²

(a) *Alkaline Egg Solution*.—Beat up a whole egg with an equal volume of distilled water. Mix an equal volume of this with an equal volume of 6.5 per cent. solution of anhydrous sodium carbonate and steam for from one-half to one hour.

(b) *Meat extract-glucose-agar*—

Distilled water	1000 c.c.
Meat extract (Liebig)	3 grams
Peptone (Witte).	10 grams
Sodium chloride (c. p.)	5 grams
Glucose	1 gram
Agar-agar	30 grams

¹ Jour. Infect. Dis., 1912, x, 134.

² U. S. Pub. Health Service, Hygiene Lab. Bull., 1913, No. 91, p. 19.

Steam at 100° C., for 3 hours to insure complete solution of the agar-agar.

Decant, or filter through cotton, and distribute in 100 c.c. flasks.

Sterilize by steam at 100° C. for an hour and a half.

For use mix one volume of *a* with 5 volumes of *b*, the latter having been completely liquefied by steam. When thoroughly mixed pour into Petri dishes, to a depth of about 3 mm. in each dish, and allow to solidify. When the medium is solid, the dishes may be placed in the incubator with the covers partly removed until the condensed vapor has evaporated. The medium should be comparatively dry before attempting to use it. When dry the plates so prepared may be stored in dust proof receptacles at 15° C. The plates may now be inoculated from the *surface* of the Benedict medium. This is best done by transferring a loopful of the Benedict culture to the surface of the solid alkaline egg-glucose-agar and distributing it over the surface with a sterile, bent-glass spreader. When thus inoculated the plates are placed in the incubator at 37°–38° C. until colonies develop.

On this medium the cholera, and the cholera-like spirilla grow luxuriantly, while the other bacteria are to some extent restrained.

The colonies of the cholera vibrios, and of those other vibrios that closely resemble it, when well developed on this medium, *i. e.*, after about twenty hours at 37°–38° C., are, to the naked eye, more opaque than those of other bacteria; under the low power lens they seem as if in the depths of the medium, are more or less hazy, are surrounded by an indistinct halo or fringe which may be in turn surrounded by a clear zone. All such colonies should be examined microscopically and from all that are composed

of curved or spiral organisms pure cultures should be made for subsequent identification.

In abortive cases of cholera the organisms may be present in the intestinal canal in very small numbers, and microscopic examination is not, therefore, of so much assistance. In these cases the adoption of one or the other of the foregoing methods is imperative.

In the foregoing suggested plans it will be observed that plates are not made in the usual way. The reason for this is the cholera spirillum being markedly aërobic develops much more readily on the surface than in the depths of the medium. For the same reason the material taken for plating from the enriching media should always be from the surfaces, without the tubes or flasks having been shaken.

It being desirable to have the colonies isolated from one another the plates should be relatively dry, that is, there should be no collection of moisture on their surfaces that would cause the colonies to become confluent. After pouring, the plates should always be kept in a dust-free incubator with their lids off until all excess of moisture is evaporated. All colonies of curved rods should be isolated in pure culture in peptone solution, and after twenty to twenty-four hours at 37° to 38° C. such cultures should be tested for the presence of indol. After giving positive indol reaction should be regarded as probably cholera spirilla.

In all doubtful cases, in which only a few curved bacilli are present, or in which irregularities in either the rate or mode of their development occur, pure cultures should be obtained as soon as possible and their virulence tested upon animals. For this purpose cultures upon agar-agar from single colonies must be made. From the surface of one of such cultures a large wire-loopful should be scraped and

broken up in about one cubic centimeter of physiological salt solution, and the suspension thus made injected by means of a hypodermic syringe directly into the peritoneal cavity of a guinea-pig of about 350 to 400 grams weight. For larger animals more material is used. If the material injected is from a *fresh* culture of the cholera organism, toxic symptoms at once appear; these have their most pronounced expression in depression of temperature, and if one follows this decline in temperature from time to time with the thermometer it will be seen to be gradual and continuous from the time of injection to the death of the animal (Pfeiffer¹), which occurs in from eighteen to twenty-four hours after the operation.

MICROSPIRA METCHNIKOVİ (GAMALEÏA), MIGULA, 1900.

SYNONYM: *Vibrio Metchnikovi*. Gamaleïa, 1888.

A spirillum that simulates very closely the comma bacillus of cholera in its morphological and cultural peculiarities, but which is still easily distinguished from it, is that described by Gamaleïa² under the name of *microspira Metchnikovi*. It was found *postmortem* in a number of fowls that had died in the poultry-market of Odessa, and the experiments of the discoverer led him to believe that it was related etiologically to the gastro-enteritis from which the chickens had been suffering.

Morphologically it appears as short, curved rods and as longer, spiral-like filaments. It is usually thicker than Koch's *microspira* and is at times *much* longer, while again it is seen to be shorter. It is usually more distinctly curved than the "comma bacillus." (Fig. 92.)

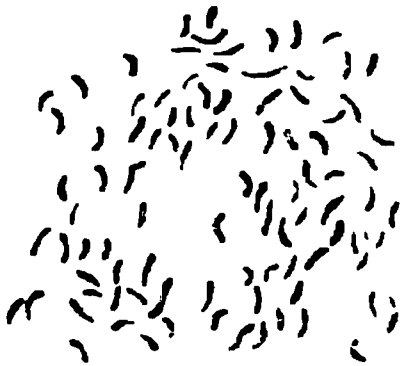
¹ Loc. cit. ² *Annales de l'Institut Pasteur*, 1888, tome ii, pp. 482, 552.

It is supplied with a single flagellum at one of its extremities, and is therefore motile.

It does not form spores.

It is aërobic.

FIG. 92



Microspira Metchnikovi from agar-agar culture, twenty-four hours old.

Its growth upon gelatin plates is usually characterized, according to Pfeiffer, by the appearance of two kinds of liquefying colonies, one strikingly like those of the Finkler-Prior organism, the other very similar to those produced by Koch's comma bacillus, though in both cases the lique-

FIG. 93



Colony of *microspira Metchnikovi* in gelatin, after thirty hours at 20° to 22° C. X about 75 diameters.

faction resulting from the growth of this organism is more energetic than that common to the spirillum of Asiatic cholera. After from twenty-four to thirty hours the medium-size colonies, when examined under a low power of the

microscope, show a yellowish-brown, ragged central mass surrounded by a zone of liquefaction that is marked by a border of delicate radii. (Fig. 93.)

In gelatin stab-cultures the growth has much the same

FIG. 94

a b c d

Stab-culture of *microspira Metchnikowi* in gelatin, at 18° to 20° C. a, after twenty-four hours; b, after forty-eight hours; c, after seventy-two hours; d, after ninety-six hours.

general appearance as that of the cholera spirillum, but is exaggerated in degree. The liquefaction is far more rapid, and the characteristic appearance of the growth is lost in from three to four days. (See a, b, c, d, Fig. 94.) Development and liquefaction along the deeper parts of the needle-

track are much more pronounced than is the case with the "comma bacillus."

Its growth on agar-agar is rapid; after twenty-four to forty-eight hours a grayish deposit appears which has a tendency to become yellowish with age.

On potato at 37° C. its growth is seen as a moist, coffee-colored patch, surrounded by a much paler zone. The whole growth is so smooth and glistening that it has somewhat the appearance of being varnished.

In bouillon it quickly causes opacity, with the ultimate production of a delicate pellicle upon the surface.

It causes liquefaction of blood-serum, the liquefied area being covered by a dense, wrinkled pellicle.

When grown in peptone solution it produces indol and coincidentally nitrites, so that the rose-colored reaction characteristic of indol is obtained by the addition of sulphuric acid alone. The production of indol by this organism is usually *greater* than that common to the comma bacillus under the same circumstances.

In milk it causes an acid reaction with coagulation of the casein. The coagulated casein collects at the bottom of the tube in irregular masses, above which is a layer of clear whey. If blue litmus has been added to the milk, the color is changed to pink in from twenty-four to thirty hours, and after forty-eight hours decolorization and coagulation occur. The clots of casein are not re-dissolved. After about a week the acidity of the milk is at its maximum, and the organisms quickly die.

It causes the red color of the rosolic-acid-peptone solution to become very much deeper after four or five days at 37° C.

It does not cause fermentation of glucose with production of gas.

It is killed in five minutes by a temperature of 50° C. (Sternberg.)

It is pathogenic for chickens, pigeons, and guinea-pigs. Rabbits and mice are affected only by very large doses.

Gamaleïa states that chickens affected with the choleraic gastro-enteritis of which this organism is the cause, are usually seen sitting quietly with ruffled feathers. They suffer from diarrhea, but there is no elevation of temperature. Hyperemia of the entire gastro-intestinal tract is seen at autopsy. The other internal organs do not, as a rule, present anything abnormal to the naked eye. The intestinal canal contains yellowish fluid with which blood may be mixed. In adult chickens the spirilla are not found in the blood, but in young ones they are usually present in small numbers.

After the introduction of a very small quantity of a culture of this organism directly into the pectoral muscle pigeons succumb in from eight to twenty hours. The most conspicuous *postmortem* lesion is found at the site of inoculation. The muscle is marked by yellow, necrotic stripes; is more or less edematous; is swollen, and contains the vibrios in enormous numbers. The intestines are usually filled with fluid contents, which may or may not be blood-stained; the walls of the intestines are often injected with blood, and occasionally markedly so. The conditions of the other internal viscera are inconstant. In fatal cases the vibrios are present in large numbers in the blood and internal organs. In pigeons that survive inoculation the organisms may be found only at the site of inoculation, or very sparingly in the blood also. These animals usually exhibit immunity from subsequent inoculations. In certain instances the results of infection are chronic; the inoculated

pectoral muscle atrophies, the pigeon loses in weight and finally dies after one or two weeks. In these cases the organisms are usually absent from the blood and internal organs, and may even be absent from the site of inoculation, or, if present, in only very small number.

Guinea-pigs usually die in from twenty to twenty-four hours after subcutaneous inoculation. At autopsy an extensive edema of the subcutaneous tissues about the seat of inoculation is seen, and there is usually a necrotic condition of the tissues in the vicinity of the point of puncture. As the blood and internal organs of both pigeons and guinea-pigs contain the vibrios in large numbers, the infection in these animals takes, therefore, the form of acute, general septicemia.

The blood-serum of both pigeons and guinea-pigs that have survived inoculation with this organism—*i. e.*, that have acquired immunity from it—is bactericidal *in vitro* for this organism. It also possesses a certain degree of immunity-conferring property, as may be demonstrated by injecting it into normal pigeons and guinea-pigs that are subsequently to be inoculated with virulent cultures.

Very old cultures of this organism in bouillon become distinctly alkaline in reaction. At this stage they contain a toxin that is markedly active for susceptible animals. This toxin is not dissolved in the fluid to any extent, but is apparently in intimate association with the proteid matters composing the bacteria.

Gastro-enteritis may be produced in both chickens and guinea-pigs by feeding them with food with which cultures of this organism have been mixed. (Gamaleïa.)

MICROSPIRA SCHUYLKILLIENSIS, ABBOTT, 1896.

SYNONYM: *Vibrio Schuyllkilliensis*, Abbott, 1896.

Abbott¹ discovered a microspira in the water of the Schuylkill River, at Philadelphia, and later, Bergey² reports the presence of the same organism, as well as several varieties that are slightly different, in the waters of the Schuylkill and Delaware rivers, along the entire city front, more especially in the effluents of the sewers.

Microspira Schuyllkilliensis is a short, rather plump "comma," often with a very decided curve, with rounded or slightly pointed ends. As usually seen it is a little shorter and thicker than the *microspira comma*, though this feature is quite variable. It is actively motile, having a single polar flagellum. It does not form spores. It stains with the ordinary aniline stains, but is negative to Gram's method.

The colonies on gelatin are sharply defined, distinctly granular, and have usually fine irregular markings, as if they were creased or folded. Sometimes they present indistinct concentric markings. As growth progresses these markings become more and more distinct and finally give to the colony a decidedly lobulated or mulberry-like appearance.

After about the third or fourth day, when liquefaction is actively in progress, the majority of the colonies lose their characteristic appearance. They are seen as irregular, ragged, granular masses lying in the centre of pits of liquefied gelatin.

In stab cultures in gelatin the appearance of the growth is essentially that of *microspira comma*, though at times it is a little more rapid in progress.

¹ Jour. of Exp. Med., 1896, i, 419.

² Ibid., 1897, ii, 535.

On meat-infusion agar-agar, neutral or slightly alkaline to phenolphthalein, growth is very rapid at the body temperature. The general character of the growth corresponds to that of *Microspira comma*.

The growth on blood serum, after twenty-four hours at body temperature, appears as a line of depression, which increases as a track of liquefaction, and later results in the more or less complete liquefaction of the medium.

Bouillon becomes uniformly clouded in twenty-four hours at the body temperature. Its reaction becomes more alkaline as growth progresses. A pellicle, at first delicate, later denser, always characterizes the growth in this medium.

Usually no visible growth occurs on a potato.

In fresh litmus-milk a slight degree of acidity is noticed after twenty-four hours at body temperature. After forty-eight hours this acidity is slightly greater, and at times the milk shows evidences of coagulation, though not always.

Microspira Schuyllkilliensis is a facultative aërobe. In fluid media under an atmosphere of carbon dioxide in sealed tubes no growth is observed.

The organism grows most luxuriantly at about 37.5° C. Growth is hardly perceptible at 10° C. It is destroyed by an exposure of five minutes to 50° C.

None of the carbohydrates are broken up with the liberation of gas.

It produces indol and at the same time reduces nitrates to nitrites.

The pathogenic properties of this organism are best seen in guinea-pigs and pigeons, both of which are uniformly susceptible. Rabbits and chickens resist relatively large doses. Mice are infected with small doses injected subcutaneously.

The most characteristic lesions follow the injection of cultures into the pectoral muscles of pigeons. At death the inoculated muscle is swollen, necrotic, and the over-lying tissues are edematous. The bacteria are found in large numbers in the vicinity of the seat of the inoculation, and in relatively small numbers in the blood and internal organs.

CHAPTER XXVII.

Study of Bacterium Anthracis, and of the Effects Produced by Its Inoculation into Animals—Peculiarities of the Organism Under Varying Conditions of Surroundings—Anthrax Vaccines—Anthrax Immune Serum.

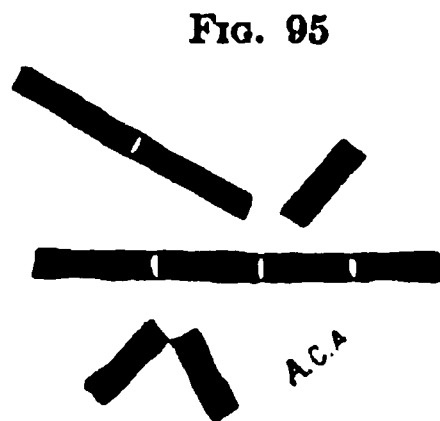
THE discovery that the blood of animals suffering from splenic fever, or anthrax, always contains minute rod-shaped bodies (Pollender, 1855; Davaine, 1863), led to a group of investigations that have not only fully familiarized us with the nature of this malady in particular, but have perhaps contributed more, incidentally, to our knowledge of bacteriology in general than studies upon any other single infective process or its causative agent.

The direct outcome of these investigations is that a rod-shaped micro-organism, now known as bacterium anthracis, is always present in the blood of animals suffering from this disease; that this organism can be obtained from the tissue of those animals in pure cultures; and that such artificial cultures of bacterium anthracis when introduced into the bodies of susceptible animals can again produce a condition identical with that found in the animal from which they were obtained. The disease is a true septicemia, and after death the capillaries throughout the body are always found to contain the typical rod-shaped organism in larger or smaller numbers.

This organism, when isolated in pure culture, is a bacterium which varies considerably in length, ranging from short rods, 2 to 3 μ in length, to longer threads, 20 to 25 μ

in length. In breadth it is from 1 to 1.25μ . Frequently very long threads, made up of several rods joined end to end, are seen.

When obtained directly from the body of an animal it is usually in the form of short rods *square at the ends*. If highly magnified, the ends are seen to be a trifle thicker than the body of the cell and somewhat indented or concave, suggestive of the joints of bamboo, peculiarities that help to distinguish it from certain other organisms that are somewhat like it morphologically. (See Fig. 95.)



Bacterium anthracis, highly magnified to show swellings and concavities at extremities of the single cells.

When cultivated artificially at the temperature of the body the bacterium of anthrax presents a series of very interesting developmental phases.

The short rods grow into long threads, which may be seen twisted or plaited together like ropes, each thread being marked by the points of juncture of the segments composing it. (Fig. 96, *a* and *b*.) In this condition it remains until alterations in its surroundings, the most conspicuous being diminution of its nutritive supply, favor the production of spores. When this stage begins changes in the protoplasm may be noticed; the bacteria become marked by irregular granular bodies, which eventually coalesce into

glistening oval spores, one of which lies in nearly every segment of the long thread, and gives to the thread the appearance of a string of shining beads. (Fig. 97.) In

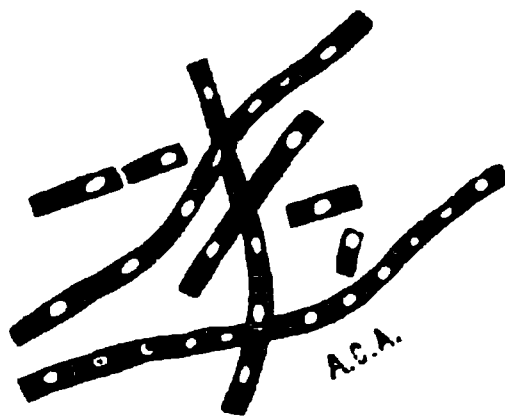
FIG. 96



Bacterium anthracis. Plaited and twisted threads seen in fresh-growing cultures. \times about 400 diameters.

this stage they remain but a short time. The chains of spores, which are held together by the remains of the cells in which they formed, become broken up, and eventually nothing but free oval spores, and here and there the remains

FIG. 97



Threads of *bacterium anthracis* containing spores. \times about 1200 diameters.

of mature bacilli which have undergone degenerative changes, can be found. In this condition the spores, capable of resisting deleterious influences, remain and, unless their sur-

roundings are altered, continue in this living, though inactive, condition for a very long time. If again placed under favorable conditions, each spore will germinate into a mature cell, and the same series of changes will be repeated until the surroundings become again gradually unfavorable to development, when spore-formation again takes place. Spore formation occurs only at temperatures ranging from 18° to 43° C.; 37.5° C. being the optimum. Under 12° C. they are not formed. This organism does not form spores in the tissues of the living animal, its usual condition at this time being

FIG. 98

Colony of bacterium anthracis on agar-agar.

that of short rods; occasionally, however, somewhat longer forms may be seen.

The bacterium of anthrax is not motile.

Colonies of this organism, as seen upon agar-agar, present a typical appearance, from which they have been likened unto the head of Medusa. From a central point, which is more or less dense, consisting of a felt-like mass of long threads irregularly matted together, the growth continues outward upon the surface of the agar-agar (Fig. 98.) It is made up of wavy bundles in which the threads are seen to lie parallel or are twisted in strands like those of a rope;

sometimes they have a plaited arrangement. (See Fig. 96.) These bundles twist and cross in all directions, and eventually disappear at the periphery of the colony. At the extreme periphery of the colonies it is sometimes possible to trace single bundles of these threads for long distances across the surface of the agar-agar. The colony itself is not circumscribed in appearance, but is more or less irregularly fringed or ragged, or scalloped. To the naked eye they look very much like minute pellicles of raw cotton that have been pressed into the surface of the agar-agar.

As the colonies continue to grow they become more and more dense and opaque, and granular and rough on the surface. When touched with a sterilized needle one experiences a sensation that suggests somewhat their matted structure. They are never moist or creamy. The bit that is taken up with the needle is always more or less ragged, suggesting a tiny particle of moist blotting paper.

The colonies on gelatin at the earliest stages also present the same wavy appearance; but this characteristic soon becomes in part destroyed by the liquefaction of the gelatin which is produced by the growing organisms. This allows them to sink to the bottom of the fluid, where they lie as irregular masses. Through the fluid portion of the gelatin may be seen small clumps of growing bacteria, which look very much like bits of cotton-wool.

In bouillon the growth is characterized by the formation of flaky masses, which also have very much the appearance of bits of raw cotton. Microscopic examination of one of these flakes reveals the twisted and plaited arrangement of the long threads.

On potato it develops rapidly as a dull, dry, granular, whitish mass, which is more or less limited to the point of

inoculation. On potato, at the temperature of the incubator, spore-formation may be easily observed.

Stab- and slant-cultures on agar-agar present in general the appearances given for the colonies, except that the growth is much more extensive. The growth is always more pronounced on the surface than down the track of the needle.

On gelatin it causes liquefaction, which begins on the surface at the point inoculated and spreads outward and downward.

It grows best with access to oxygen, and very poorly when the supply of that gas is interfered with.

Under favorable conditions of aëration, nutrition, and temperature its growth is rapid.

Under 12° C. and above 45° C. no growth occurs. Its optimum temperature is that of the body, viz., 37°–38° C.

The spores of bacterium anthracis are very resistant to heat, though the degree of resistance varies with spores of different origin. Von Esmarch found that anthrax spores from some strains were readily killed by an exposure of one minute to the temperature of steam, whereas spores from others resisted this temperature longer, in some cases as long as twelve minutes.

STAINING.—Anthrax bacteria stain readily with the ordinary aniline dyes. In tissues their presence may also be demonstrated by the ordinary aniline staining-fluid or by Gram's method. They may also be stained in tissues with a strong watery solution of dahlia, after which the sections are decolorized in 2 per cent. sodium carbonate solution, washed in water, dehydrated in alcohol, cleared in xylol, and mounted in balsam. This leaves the bacilli stained, while the tissues containing them are decolorized;

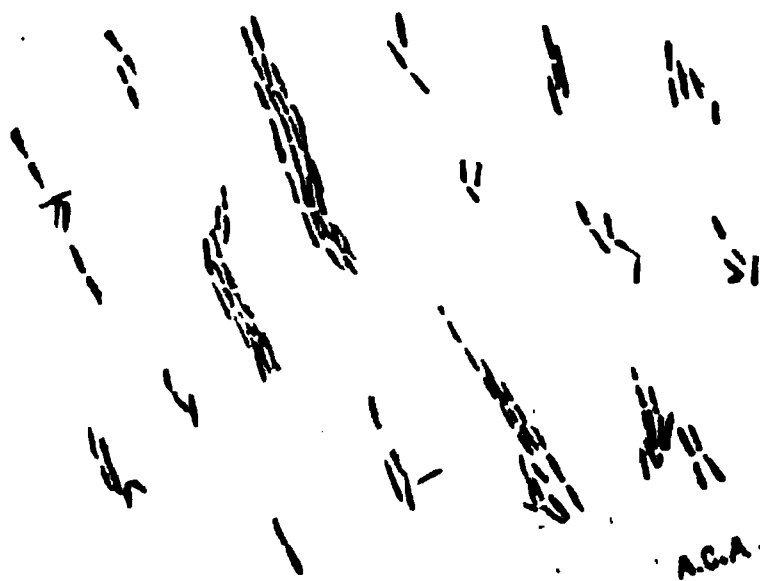
or the latter may be stained a contrast-color—with eosin, for example—after dehydration in alcohol and before clearing in xylol. In this case they must be washed again in alcohol before using the xylol. In a preparation treated in this way the rod-shaped organisms are of a purple color, and will be seen in the capillaries of the tissues, while the tissues themselves are of a pale rose color.

Inoculation into Animals.—Introduce into the subcutaneous tissues of the abdominal wall of a guinea-pig or rabbit a portion of a pure culture of *bacterium anthracis*. The animal usually succumbs in from thirty-six to forty-eight hours. Little or no reaction at the immediate point of inoculation will be noticed; but beyond this, extending for a long distance over the abdomen and thorax, the tissues will be markedly edematous. Here and there, scattered through this edematous tissue, small ecchymoses will be seen. The underlying muscles are pale in color. Inspection of the internal viscera reveals no very marked macroscopic changes except in the spleen. This is enlarged, dark in color, and soft. The liver may present the appearance of cloudy swelling; the lungs may be red or pale red in color; the heart is usually filled with blood. No other changes can be seen by the naked eye.

Prepare cover-slip preparations from the blood and other viscera. They will all be found to contain short rods in large numbers. Nowhere can spore-formation be detected. Upon microscopic examination of sections of the organs which have been hardened in alcohol the capillaries are seen to be filled with the bacteria; in some places closely packed in large numbers, at other points fewer in number. Usually they are present in largest numbers in those tissues having the greatest capillary distribution and at those points at

which the circulation is slowest. They are uniformly distributed through the spleen. The glomeruli of the kidneys and the capillaries of the lungs are frequently packed with them. The capillaries of the liver contain them in large numbers. (Fig. 99.) Hemorrhages, probably due to rupture of capillaries by the mechanical pressure of the bacteria which are developing within them, not uncommonly occur. When these occur in the mucous membranes of the alimentary tract the blood may escape through the mouth

FIG. 99



Bacterium anthracis in liver of mouse. \times about 450 diameters. Bacteria stained by Gram's method; tissue stained with Bismarck-brown.

or anus; when in the kidneys, through the uriniferous tubules.

Cultures from the different organs or from the edematous fluid about the point of inoculation result in growth of *bacterium anthracis*.

The amphibia, dogs, and the majority of birds are not susceptible to this disease. Rats are difficult to infect. Rabbits, guinea-pigs, white mice, gray house-mice, sheep, and cattle are susceptible. Infection may occur either

through the circulation, through the air-passages, through the alimentary tract, or, as we have just seen, through the subcutaneous tissues.

Protective Inoculation.—The most noteworthy application of artificially prepared living vaccines to the protection of animals from infection is seen in connection with anthrax in sheep and in bovines.

By a variety of procedures the virulent anthrax bacterium may be in part or totally robbed of its pathogenic properties. It is through the very mild constitutional disturbance caused in animals vaccinated with such weakened cultures that protection is often afforded against the severer, frequently fatal, form of the infection.

Without reviewing the various methods that have been employed for attenuating the virulence of this organism to a degree suitable for protective vaccination, it will suffice to say that the most satisfactory results have been obtained by the classical method of Pasteur. This comprehends the long-continued cultivation (ten to thirty days) at a temperature of from 42° to 43° C. In this procedure the spore-free, virulent *bacterium anthracis*, obtained directly from the blood of a recently dead animal, is brought at once into sterile nutrient bouillon in about twenty test-tubes, which are immediately placed in an incubator that is carefully regulated to maintain a temperature of 42.5° C. There should not be a fluctuation of over 0.1° C.

After about a week a tube is removed from the incubator on each successive day and its virulence tested at once on animals. The degree of attenuation experienced by the cultures grown under these circumstances is determined by tests upon rabbits, guinea-pigs, and mice. The first culture removed may or may not kill rabbits, the most resistant

of the three animals used for the test, while it will certainly kill the guinea-pigs and mice; after another two or three days rabbits will no longer succumb to inoculation with the culture last removed from the incubator, while no diminution will as yet be noticed in its pathogenesis for the other two species. After four to seven days more a culture may be encountered that kills only mice, the guinea-pigs escaping; while ultimately, if the experiment be continued, a degree of attenuation may be reached in which the organism has not even the power of killing a mouse, though it still retains its vitality. Investigation of these attenuations shows them to possess all the characteristics of enfeebled anthrax bacteria; they grow slowly and less vigorously when transplanted; they do not form spores when exposed to a high temperature; and microscopically they present evidences of degeneration. When introduced beneath the skin of animals they disseminate but slightly beyond the site of inoculation, and do not, as a rule, cause the general septicemia that occurs in susceptible animals inoculated with normal cultures of this organism. In the practical employment of these attenuated cultures for protective purposes two vaccines are employed. These were designated by Pasteur as "first" and "second" vaccines. The "first" is the one that killed only the mice in the preliminary tests; while the "second" is that which killed both mice and guinea-pigs, but failed to kill the rabbit. When larger animals, such as sheep or cattle, are to be protected by vaccination with these vaccines, a subcutaneous inoculation of about 0.3 c.c. of the first vaccine is usually given. This should be practically without noticeable effect, causing neither rise of body-temperature nor other constitutional or local symptoms. After a period of about two weeks the second vaccine

is injected in the same way; this may or may not cause disturbance. In the event of its doing so the symptoms are rarely alarming, and, if the vaccines have been properly prepared and tested before use, all symptoms disappear within a short time after the injection.

In the large majority of cases sheep, bovines, horses, and mules may be safely protected against anthrax by the careful practice of this method.

Sobernheim¹ found that it was possible to bring about a high degree of immunity against bacterium anthracis by means of the vaccines 1 and 2 of Pasteur, with subsequent inoculations of virulent organisms. He employed the serum of animals thus immunized in the *treatment* of sheep that had been injected with highly virulent anthrax bacteria. Five sheep were treated in this way, and all of them recovered with only slight rise in temperature and moderate infiltration at the point of injection, while control animals died very promptly.

He further² reports an improvement on the method of *protective inoculation* against anthrax in which he uses a combination of anthrax vaccines and immune serum, in which the results are far more satisfactory than with the anthrax vaccines alone. He states that this new method has the following advantages over the Pasteur method: (1) That the immunization can be carried out without losing any of the animals; (2) that it can be completed in one day; (3) that stronger and more active cultures can be employed and therefore a more durable immunity obtained; and (4) that the serum alone can be employed as a curative agent.

¹ Berliner klin. Wochenschr., 1897.

² Ibid., 1902, p. 516.

Anthrax Immune Serum.—Sanfelice¹ experimented with the serum of dogs that had been immunized from anthrax bacteria. This serum possessed immunizing and curative properties, as shown by experiments upon animals. He had an opportunity of trying the serum, with favorable results, upon a man who had contracted anthrax. The total amount of serum employed was 56 cubic centimeters. There was no reaction at the point of injection of the serum. The therapeutic effect of the administration of serum was a general improvement in the symptoms, marked fall of the temperature on the second, and complete apyrexia on the third day. The effect on the local anthrax lesion manifested itself in reduction and, finally, disappearance of the edema, followed first by an increased swelling of the glands, which decreased again subsequently. He states that the serum treatment should be continued not only till the temperature has fallen to normal and a diminution of the edema is apparent, but also until there is marked reduction in the size of the swollen lymph-glands.

Sclavo² immunized a number of animals, principally sheep and goats, with the two vaccines of Pasteur, followed by repeated injections of increasing quantities of virulent cultures. By this means he obtained an immune serum which had protective as well as curative properties when tested upon guinea-pigs and rabbits.

Cicognani³ employed Sclavo's immune serum on 12 persons suffering from various grades of anthrax infection, some of the cases being severe infections in which the prognosis would otherwise have been very unfavorable. The

¹ Centralblatt f. Bacteriologie, Originale, 1902, Bd. xxxiii.

² Bulletin de l'Institut Pasteur, T. I., 1903, p. 305.

³ Centralblatt f. Bacteriologie, 1902, ref. Bd. 31, p. 725.

duration of the disease was always very much shortened and all recovered.

Lazaretti¹ reports 23 cases of human infection with bacterium anthracis in which Sclavo's immune serum was employed with recovery in each case. Another patient, suffering from chronic alcoholism and malaria, did not recover.

Experiments.—Prepare three cultures of bacterium anthracis—one upon gelatin, one upon agar-agar, and one upon potato. Allow the gelatin culture to remain at the ordinary temperature of the room, place the agar-agar culture in the incubator, and the potato culture at a temperature not above 18° to 20° C. Prepare cover-slips from each from day to day. What differences are observed?

Prepare two potato cultures of bacterium anthracis. Place one in the incubator and maintain the other at a temperature of from 18° to 20° C. Examine them each day. Do they develop in the same way?

From a fresh culture of bacterium anthracis, in which spore-formation is not yet begun (which is the surest source from which to obtain *non-spore-bearing* anthrax bacteria?), prepare a hanging-drop preparation; also a cover-slip preparation in the usual way and stain it with a strong gentian-violet solution; and another cover-slip preparation which is to be drawn through a flame twelve to fifteen times, stained with aniline gentian-violet, washed in iodine solution and then in water. Examine these microscopically. Do they all present the same appearance? To what are the differences due?

¹ Deutsche Vierteljahrsschrift f. öffentliche Gesundheitspflege, 1903, Bd. xxxv, Supplement, p. 253.

Do the anthrax threads, as seen in a fresh, growing, hanging drop, present the same morphological appearance as when dried and stained upon a cover-slip? How do they differ?

Liquefy a tube of agar-agar, and when it is at the temperature of 40° to 43° C. add a very minute quantity of an anthrax culture which is far advanced in the spore-stage. Mix it thoroughly with the liquid agar-agar and from this prepare several hanging drops under strict antiseptic precautions, using the fluid agar-agar for the drops instead of bouillon or salt-solution. Select from among these preparations that one in which the smallest number of spores are present. Under the microscope observe the development of a spore into a mature cell. Describe carefully the developmental stages.

Prepare a 1 : 1000 solution of carbolic acid in bouillon. Inoculate this with virulent anthrax spores. If no development occurs after two or three days at the temperature of the thermostat, prepare a solution of 1 : 1200, and continue until the point is reached at which the amount of carbolic acid present *just* permits of the development of the spores. When the proper dilution is reached prepare a dozen of such tubes and inoculate one of them with virulent anthrax spores. As soon as development is well advanced transfer a loopful from this tube into a second of the carbolic acid tubes; when this has developed, then from this into a third, etc. After five or six generations have been treated in this way study the spore-production of the organisms in that tube. If it is normal, continue to inoculate from one carbolic acid tube to another, and see if it is possible by this

means to influence in any way the production of spores by the organism with which you are working. What is the effect, if any?

Prepare two bouillon cultures, each from *one drop of blood of an animal dead of anthrax*. (Why from the blood of an animal and not from a culture?) Allow one of them to grow for from fourteen to eighteen hours in the incubator; allow the other to grow at the same temperature for three or four days. Remove the first tube after the time mentioned and subject it to a temperature of 80° C. for thirty minutes. At the end of this time prepare four plates from it. Make each plate with one drop from the heated bouillon culture. At the end of three or four days treat the second tube in identically the same way. How do the number of colonies which develop from the two cultures compare? Was there any difference in the time required for their development on the plates?

From a potato culture of bacterium anthracis which has been in the incubator for three or four days scrape away the growth and carefully break it up in 10 c.c. of sterilized physiological salt-solution. The more thoroughly it is broken up the more accurate will be the results of the experiment. Place this in a bath of boiling water, and at the end of one, three, five, seven, and ten minutes make plates upon agar-agar each with one loopful of the contents of this tube. Are the results on the plates alike?

Determine the exact time necessary to sterilize objects, such as silk or cotton threads, on which anthrax spores have been dried, by the steam method and by the hot-air method.

Prepare a bouillon culture from the blood of an animal just dead of anthrax. After this has been in the incubator for from three to four hours subject it to a temperature of 55° C. for ten minutes. At the end of this time make plates from it and also inoculate a rabbit subcutaneously with it. What are the results? Are the colonies on the plates in every way characteristic?

Inoculate six Erlenmeyer flasks of sterile bouillon, each containing about 35 c.c. of the medium, from the blood of an animal just dead of anthrax.

Place these flasks in the incubator at a temperature of 42.5° C. At the end of five, ten, fifteen, twenty, twenty-five, or more days remove a flask. Label each flask as it is taken from the incubator with the exact number of days that it has been at the temperature of 42.5 C. Study each flask carefully, both in its culture-peculiarities and in its pathogenic properties when employed on animals.

Are these cultures identical in all respects with those that have been kept at 37° C.?

If they differ, in what respect is the difference most conspicuous?

Should any of the animals survive the inoculations made from the different cultures in the foregoing experiment, note carefully which one it is, and after ten to twelve days repeat the inoculation, using the same culture; if it again survives, inoculate it with the culture preceding the one just used in the order of removal from the incubator; if it still survives, inoculate it with virulent anthrax. What is the result? How is the result to be explained? Do the cultures which were made from these flasks at the time of their removal from the incubator act in the same way toward

animals as the organisms growing in the flasks? Is the action of each of these cultures the same for mice, guinea-pigs, and rabbits?

Prepare a 2 per cent. solution of sulphuric acid in distilled water; suspend in this a number of anthrax spores; at the end of three, six, and nine days at 35° C. inoculate both a guinea-pig and a rabbit. Prepare cultures from this suspension on the third, sixth, and ninth days; when the cultures have developed inoculate a rabbit and a guinea-pig from the culture made on the ninth day. Should the animals survive, inoculate them again after three or four days with a culture made on the sixth day. Do the results appear in any way peculiar?

CHAPTER XXVIII.

The Nitrifying Bacteria—The Bacillus of Tetanus—The Bacillus of Malignant Edema—The Bacillus of Symptomatic Anthrax—Bacterium Welchii—Bacillus Sporogenes.

THE NITRIFYING BACTERIA.

By the employment of bacteriological methods in the study of the soil much light has been shed upon the cause and nature of the interesting and momentous biological phenomena there constantly in progress. Of these, the one of the greatest importance comprises those changes that accompany the widespread process of disintegration and decomposition, to which reference has already been made. (See Chapter I.) This resolution of dead complex organic compounds into simpler structures assimilable as food by growing vegetation is dependent upon the activities of bacteria located in the superficial layers of the ground. It is not a simple process, brought about by a single, specific species of bacteria, but represents a sequence of events each of which probably results from the activities of different species or groups of species, working alone or together. Our knowledge upon the subject does not permit of the following in detail of the manifold alterations undergone by dead organic material, but we do know that much of it is ultimately converted into inorganic matters and that carbon dioxide, ammonia and water are always conspicuous end products. When the process of decomposition occurs in the soil it does not cease at this point, but we find still further altera-

tions—alterations having to do more particularly with the ammonia. This change in ammonia is characterized by the products of its oxidation, viz., by the formation of nitrous and nitric acids and their salts; this is not a result of the direct action of atmospheric oxygen upon the ammonia, but occurs through the instrumentality of a special group of saprophytes known generically as the *nitrifying* organisms. They are found in the most superficial layers of the ground, and though more common in some places than in others, they are, nevertheless, present over the entire surface of the earth. The most conspicuous example of the functional activity of this group of soil organisms is seen in the immense saltpeter-beds of Chili and Peru, where, by the activities of these microscopic plants, nitrates are produced from the ammonia of the fecal evacuations of sea-fowls and from decomposing seaweeds in such enormous quantities as to form a source of supply of crude saltpeter for the commercial world. A more familiar example is seen in the decomposition and subsequent nitrification of the organic matters of sewage and other fluid wastes of organic nature in the process of purification by percolation through the soil, a process in which it is possible to follow, by chemical means, the organic matters from their condition as such to their ultimate conversion into ammonia, nitrous and nitric acids. In fact, the same breaking down and building up, resulting ultimately in nitrification, occurs in all nitrogenous matters that are deposited upon the soil and allowed to decay. It is largely through this means that growing vegetation obtains the nitrogen necessary for the nutrition of its tissues, and when viewed from this standpoint we appreciate the importance of this process to all life, animal as well as vegetable, upon the earth.

Under special circumstances there occurs in the soil a process the reverse of nitrification, that is, a reduction of nitrates and nitrites to lower compounds and ultimately to free gaseous nitrogen. This so-called "denitrification," while the result of bacterial activity is not dependent upon such specific varieties of bacteria as is nitrification. For instance, true denitrification is known to be an attribute of *bacillus coli communis*, of *bacillus fluorescens liquefaciens*, of *bacillus pyocyaneus*, and of *bacillus typhosus*. While this group of species ordinarily develop under free access of oxygen they can develop without it and secure their necessary oxygen from such oxides of nitrogen as nitrates and nitrites, thus reducing them. It seems probable that certain products of bacterial growth have also a reducing action on soil nitrates. Denitrification occurs most often and most actively in soils containing an excess of undecomposed organic matter.

In addition to nitrification and denitrification there is seen in the soil a phenomenon resulting in "nitrogen fixation." In some instances this results from the symbiotic activities of bacteria and higher plants, in others it appears to be peculiar to certain definite species of bacteria acting alone. While a discussion of the extreme agricultural importance of these phenomena would be of great interest, yet this is scarcely the place to undertake it.¹

The unusual nature of the nitrifying bacteria, demanding as they do special methods for their cultivation, renders them of sufficient technical interest to justify—for purposes of illustration—a more or less detailed description of one of them.

These very important and interesting nitrifying organisms,

¹ See *Bacteria in Relation to Country Life*, by Lipman-MacMillan, 1911.

of which there appear to be several, evade all efforts to isolate them from the soils and to cultivate them by the methods commonly employed in bacteriological work. They can be successfully studied only through the employment of special media.

The organism generally known as the nitro-monas of Winogradsky is a short, oval, and frequently almost spherical cell. It reproduces by segmentation as usual for bacteria, but there is little tendency for the daughter-cells to adhere together or to form chains. In cultures they are commonly massed together, by a gelatinous material, in the form of zoöglea. It does not form spores, and is probably not motile, though Winogradsky believes he has occasionally detected it in active motion. As has been stated, it does not grow upon ordinary nutrient media, and cannot, therefore, be isolated by the means commonly employed to separate different species of bacteria. The most astonishing property of this organism is its ability to grow and perform its specific fermentative function in solutions devoid of organic matter. It is believed to be able to obtain its necessary carbon from carbon dioxide. For its isolation and cultivation Winogradsky recommends the following solution:

Ammonium sulphate	1 gram
Potassium phosphate	1 gram
Pure water	1000 c.c.

To each flask containing 100 c.c. of this fluid is added from 0.5 to 1 gram of basic magnesium carbonate suspended in a little distilled water and sterilized by boiling. One of the flasks is then to be inoculated with a minute portion of the soil under investigation, and after four or five days a small portion is to be withdrawn, by means of a *capillary* pipette,

from over the surface of the layer of magnesium carbonate and transferred to a second flask, and similarly after four or five days from this to a third flask, and so on. As this medium does not offer conditions favorable to the growth of bacteria requiring organic matter for their development, those that were originally introduced with the soil quickly disappear, and ultimately only the nitrifying organisms remain. These are seen as an almost transparent film attached to the clumps and granules of magnesium carbonate on the bottom of the flask.

For their cultivation upon a solid medium Winogradsky employs a mineral gelatin, the gelatinizing principle of which is silicic acid. A solution of from 3 to 4 per cent. of silicic acid in distilled water, and having a specific gravity of 1.02, remains fluid and can be preserved in flasks in this condition. (Kühne.) Gelatinization occurs after the addition of certain salts to such a solution, and will be more or less complete according to the proportion of salts added. The salts that have given the best results and the method of mixing them are as follows:

a {	Ammonium sulphate	0.40 gram
	Magnesium sulphate	0.05 gram
	Calcium chloride		trace.
b {	Potassium phosphate	0.10 gram
	Sodium carbonate	0.6 to 0.90 gram
	Distilled water	100.00 c.c.

The sulphates and chloride (*a*) are mixed in 50 c.c. of the distilled water, and the phosphate and carbonate (*b*) in the remaining 50 c.c., in separate flasks.

Each flask is then sterilized with its contents, which after cooling are mixed; the mixture representing the solution of mineral salts is to be added to the silicic acid, little by

little, until the proper degree of consistency is obtained (that of ordinary nutrient gelatin). This part of the process is best conducted in a culture-dish. If it is desired to separate the colonies, as in an ordinary plate, the inoculation and mixing of the material introduced must be done before gelatinization is complete; if the material is to be distributed over only the surface of the medium, then the mixture must first be allowed to solidify.

By the use of the silicate-gelatin Winogradsky has isolated from the gelatinous film in the bottom of fluids undergoing nitrification a bacillus which he believes to be associated with the nitro-monas in the nitrifying process.

Our knowledge of these organisms is as yet too imperfect to permit of a complete description. What has been said will serve to indicate the direction in which further studies of the subject should be prosecuted. (For further details, the reader is referred to the original contributions and to current literature on the subject.¹)

In addition to the bacteria concerned in the various transformation of nitrogen, there are occasionally present in the soil micro-organisms possessing disease-producing properties. Conspicuous among these may be mentioned the bacillus of malignant edema (*vibrion septique* of the French), the bacillus of tetanus, and the bacillus of symptomatic anthrax (*Rauschbrand* (Ger.); *charbon symptomatique* (Fr.)). It is

¹ Winogradsky, Annales de l'Institut Pasteur, 1890, tome iv; 1891, tome v.

Jordan and Richards, Report of State Board of Health of Massachusetts Purification of Sewage and Water, 1890, ii, 864.

Frankland, G. C. and P. F., Proceedings of Royal Society, London, 1890, vol. xlvii.

Winogradsky and Omeliansky, Ueber den Einfluss der organischen Substanzen auf der arbeit der nitrifizierenden Mikroben, Centralblatt für Bakteriologie, 1899, Abt. ii, Bd. v, S. 329.

sometimes due to the presence of one or the other of these organisms that wounds to which soil has had access (crushed wounds from the wheels of cars or wagons, wounds received in agricultural work, gunshot wounds, etc.) are followed by such grave consequences.

BACILLUS TETANI, NICOLAIER, 1884.

In 1884 Nicolaier produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden-earth, and demonstrated that the pus produced at the point of inoculation was capable of reproducing the disease in other mice and rabbits. He did not succeed in isolating the organism in pure culture. In 1884 Carle and Rattone, and in 1886 Rosenbach, demonstrated the infectious nature of tetanus as it occurs in man by producing the disease in animals by inoculating them with secretions from the wounds of individuals affected with the disease. In 1889 Kitasato obtained the bacillus of tetanus in pure culture, described his method of obtaining it and detailed its biological peculiarities as follows:

Method of Obtaining.—Inoculate several mice subcutaneously with secretions from the wound of a case of typical tetanus. This material usually contains not only tetanus bacilli, but other organisms as well, so that at autopsy, if tetanus results, there may be more or less supuration at the seat of inoculation in the mice. In order to separate the tetanus bacillus from the others that are present the pus is smeared upon the surface of several slanted blood-serum or agar-agar tubes and placed at 37° to 38° C. After twenty-four hours all the organisms will have developed, and microscopic examination will usually

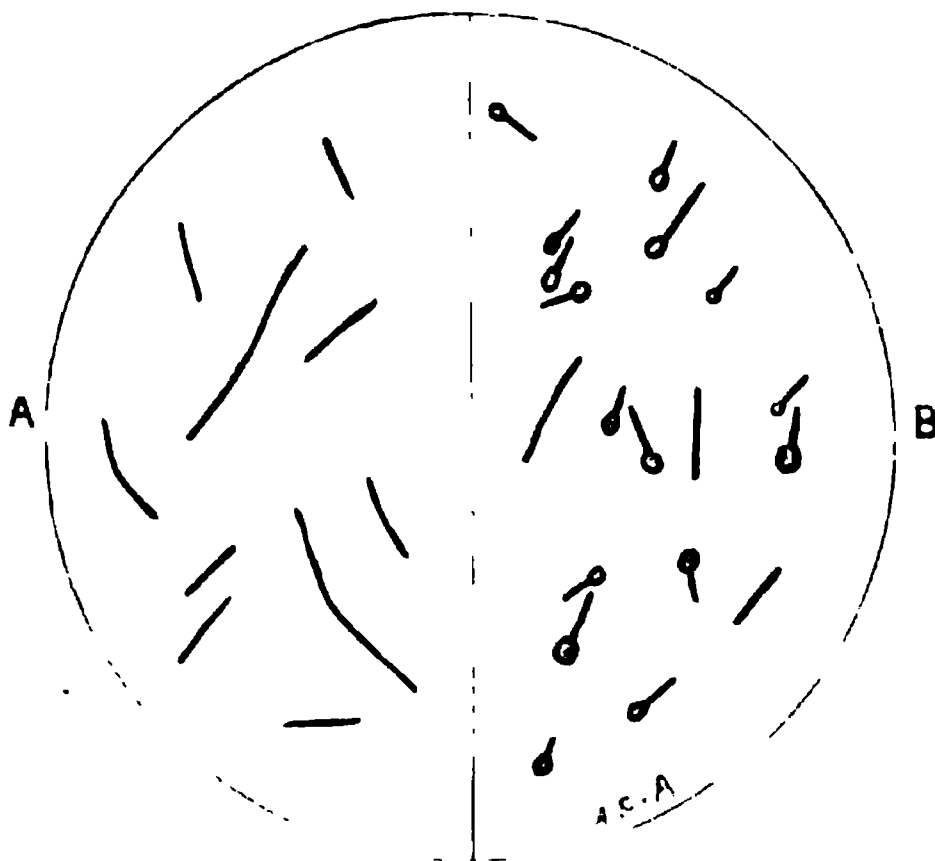
reveal the presence of a few tetanus bacilli, recognizable by their shape, viz., that of a small pin, with a spore representing the head. After forty-eight hours at 38° C. the culture is subjected to a temperature of 80° C. in a water-bath for from three-quarters to one hour. At the end of this time series of plates or Esmarch tubes of slightly alkaline gelatin are made with very small amounts of the culture and kept in an atmosphere of hydrogen (see page 224). They are then kept at from 18° to 20° C., and at the end of about a week the tetanus bacillus begins to appear in the form of colonies. After about ten days the colonies should not only be examined microscopically, but each colony that has developed in the hydrogen atmosphere should be obtained in pure culture and again grown under the same conditions. The colonies that grow only without oxygen, and which are composed of the pin-shaped organisms, must be tested upon mice. If they represent growth of the tetanus bacillus, the typical clinical manifestations of the disease will be produced in these animals.

In obtaining the organism from the soil much difficulty is experienced. Here are encountered a number of spore-bearing organisms that are facultative in their relation to oxygen, and are therefore very difficult to eliminate; and there is, moreover, one in particular that, like the tetanus bacillus, forms a polar spore. This spore is, however, much more oval than that of the tetanus bacillus, and gives to the organism containing it more the shape of a javelin (or *clostridium*, properly speaking) than that of a round-headed *pin*, the characteristic shape of the spore-bearing tetanus organism. It is non-pathogenic, and grows both with and without oxygen, and should, consequently, not be mistaken for the latter bacillus. It must also be borne

in mind that there are occasionally present in the soil still other bacilli which form polar spores, and which, when in this stage, are almost identical in appearance with the tetanus bacillus; but they will usually be found to differ from it in their relation to oxygen, and they are also without disease-producing properties.

Morphology.—In the vegetating stage it is a slender rod with rounded ends. It may appear as single rods, or, in

FIG. 100



Bacillus tetani. A, vegetative stage; B, spore-stage, showing pin-shapes.

cultures, as long threads. It is motile, though not actively so. The motility is rendered somewhat more conspicuous by examining the organism upon a warm stage.

At the temperature of the body it rapidly forms spores. These are round, thicker than the cell, and usually occupy one of its poles, giving to the rod the appearance of a small pin. (Fig. 100.) When in the spore-stage it is not motile.

It is stained by the ordinary aniline staining-reagents. It retains the color when stained by Gram's method.

FIG. 101

Cultural Peculiarities.—It is an obligate anaërobe, and cannot be brought to development under access of oxygen. It thrives in an atmosphere of pure hydrogen, but not in one of carbonic acid.

It grows in ordinary nutrient gelatin and agar-agar of a slightly alkaline reaction. Gelatin is slowly liquefied, with the coincident production of a small amount of gas. Blood-serum is not liquefied by its growth.

The addition to the media of from 1.5 to 2 per cent. of glucose, 0.1 per cent. of indigo-sodium sulphate, or 5 per cent. by volume of blue litmus tincture favors its growth.

It grows well in alkaline bouillon under an atmosphere of hydrogen.

Under artificial conditions it may be cultivated through numerous generations without loss of virulence.

Appearance of the Colonies.—Colonies of *bacillus tetani* on gelatin under an atmosphere of hydrogen have, in their early stages, somewhat the appearance of the colonies of the common *bacillus subtilis* in their earliest stages, viz., they have a

Colonies of the tetanus bacillus four days old, made by distributing the organisms through a tube nearly filled with glucose-gelatin. Cultivation in an atmosphere of hydrogen. (From Fränkel and Pfeiffer)

dense, felt-like centre surrounded by a fringe of delicate radii. The liquefaction is so slow that the appearance is retained for a relatively long time, but eventually becomes altered. In very old colonies the entire mass is made up of a number of distinct threads that give it the appearance of a common mould. (See Fig. 101.)

In *stab-cultures* made in tubes about three-quarters filled with gelatin growth begins at about 1.5 to 3 cm. below the surface, and gradually assumes the appearance of a cloudy, linear mass, with prolongations radiating into the gelatin from all sides. Liquefaction with coincident gas-production results, and may reach almost to the surface of the gelatin.

Relation to Temperature and to Chemical Agents.—It grows best at a temperature of from 36° to 38° C.; gelatin cultures kept at from 20° to 25° C. begin to grow after three or four days. In an atmosphere of hydrogen at from 18° to 20° C. growth does not usually occur before one week. No growth occurs below 14° C. At the temperature of the body spores are formed in cultures in about thirty hours, whereas in gelatin cultures at from 20° to 25° C. they do not usually appear before a week, when the lower part of the gelatin is quite fluid.

Spores of the tetanus bacillus when dried upon bits of thread over sulphuric acid in the desiccator and subsequently kept exposed to the air, retain their vitality and virulence for a number of months. Their vitality is not destroyed by an exposure of one hour to 80° C.; on the other hand, an exposure of five minutes to 100° C. in the steam sterilizer kills them. They resist the action of 5 per cent. carbolic acid for ten hours, but succumb when exposed to it for fifteen hours. In the same solution, plus 0.5 per cent. of hydrochloric acid, they are no longer active after

two hours. They are killed when acted upon for three hours by corrosive sublimate, 1 : 1000, and in thirty minutes by the same solution plus 0.5 per cent. of hydrochloric acid.

Action upon Animals.—After subcutaneous inoculation of mice with minute portions of a pure culture of this organism tetanus develops in twenty-four hours and ends fatally in from two to three days. Rats, guinea-pigs, and rabbits are similarly affected, but only by larger doses than are required for mice, the fatal dose for a rabbit being from 0.3 to 0.5 c.c. of a well-developed bouillon culture. The period of incubation for rats and guinea-pigs is twenty-four to thirty hours, and for rabbits from two to three days. Pigeons are but slightly, if at all, susceptible.

The tetanic convulsions always appear first in the parts nearest the seat of inoculation, and subsequently become general.

At autopsies upon animals that have succumbed to inoculations with *pure cultures*¹ of *bacillus tetani* there is little to be seen by either macroscopic or microscopic examination, and cultures from the site of inoculation are often negative in so far as finding the tetanus bacillus is concerned. At the site of inoculation there is usually only a hyperemic condition. In uncomplicated cases there is no suppuration. The internal organs do not present any macroscopic change, and culture-methods of examination show them to be free from bacteria. The death of the animal results from the absorption of a soluble poison, either produced by the bacteria at the site of inoculation or, which seems more probable, produced by the bacteria *in the culture* from which they are

¹ Animals and human beings that have become infected with this organism in the ordinary way commonly present a condition of suppuration at the site of infection; this is not due, however, to the tetanus bacillus, but to other bacteria that gained access to the wound at the time of infection.

obtained and introduced with them into the tissues of the animal at the time of inoculation. In support of the latter hypothesis; mice have been inoculated with pure cultures of this organism; after one hour the point at which the inoculation was made was excised and the tissues cauterized with a hot iron; notwithstanding the short time during which the organisms were in contact with the tissues and the subsequent radical treatment, the animals died after the usual interval and with the typical symptoms of tetanus.

The poison produced by the tetanus bacillus, and to which the symptoms of the disease are due, has been isolated and subjected to detailed study; some of its toxic peculiarities, as given by Kitasato, are as follows:¹

“When cultures of this organism are robbed of their bacteria by filtration through porcelain the filtrate contains the soluble poison, and is capable, when injected into animals, of causing tetanus.

“Inoculations of other animals with bits of the organs of the animal dead from the action of the tetanus toxin produce no result; but similar inoculations with the blood or with the serous exudate from the pleural cavity always result in the appearance of tetanus. The poison is, therefore, largely present in the circulating fluids.

“The greatest amount of poison is produced by cultivation in fresh neutral bouillon of a very slightly alkaline reaction.

“The activity of the poison is destroyed by an exposure of one and one-half hours to 55° C.; of twenty minutes to 60° C.; and of five minutes to 65° C.

“By drying at the temperature of the body under access of air the poison is destroyed; but by drying at the ordinary

¹ Zeitschrift für Hygiene, 1891, Bd. x, S. 267.

temperature of the room, or at this temperature in the desiccator over sulphuric acid, it is not destroyed.

"Diffuse daylight diminishes the intensity of the poison. Its intensity is preserved when kept in the dark.

"Direct sunlight robs it of its poisonous properties in from fifteen to eighteen hours.

"Its activity is not diminished by diluting a fixed amount with water or nutrient bouillon.

"Mineral acids and strong alkalies lessen its intensity."

The chemical nature of this poison is not positively known, but according to the observations of Brieger and Cohn¹ its designation of "Toxalbumen" is a misnomer, for its reactions do not warrant its classification with the albumins in the sense in which the word is commonly used. When obtained in a pure, concentrated form, its toxic properties are seen to be altered by acids, by alkalies, by sulphuretted hydrogen, and by temperatures above 70° C. Even when carefully protected from light, moisture, and air, it gradually becomes diminished in strength, doubtless due to the formation of "toxons" and "toxoids," analogous to those observed by Ehrlich in deteriorating diphtheria toxin. When freshly prepared by the methods of the authors just cited, its potency is almost incredible 0.00005 milligrams being sufficient to cause fatal tetanus in a mouse weighing fifteen grams.

The studies of Madsen² demonstrate it to consist of two physiologically distinct intoxicating compounds; the one, a solvent of erythrocytes—a "tetanolysin;" the other, a specific irritant which, through its influence upon the central

¹ Zeitschrift für Hygiene and Infektionskrankheiten, 1893, Bd. xv, S. 1.

² Ueber Teanolysin, Zeitschrift für Hygiene und Infektionskrankheiten, 1899, Bd. xxxii, S. 214.

nervous system,¹ accounts for the phenomena by which tetanus is characterized; to this latter the designation "tetanospasmin" is given. Madsen's observations, furthermore, confirm the deductions of Ehrlich concerning the molecular structure of bacterial toxins in general, to the effect that the molecule of tetanolysin, like that of diphtheria toxin, is a complex of at least two physiologically unlike groups; the one, characterized by its marked combining tendencies (for antitoxin), the so-called haptophore group; the other, distinguished for its intoxicating quality, the so-called toxophore group.

Tetanus Antitoxin.—The principles involved in the induction of the antitoxic state against diphtheria are likewise applicable to tetanus; in fact, the fundamental observations upon the generation of antitoxin in the living animal body were made in the course of studies on tetanus; they were subsequently applied to the study of diphtheria, with the results already noted. It is needless to enter here upon the details essential to the production of tetanus antitoxin; to all intents and purposes, they are identical with those given in the section on diphtheria. Briefly stated, animals may be rendered immune from tetanus by the repeated injection of gradually increasing non-fatal doses of tetanus toxin; when immunity is established, the circulating blood contains a body, antitoxin, that combines directly with tetanus toxin in a test-tube, and thereby renders it physiologically inactive (non-intoxicating); and the serum of the immune animal is not only capable of protecting non-immune, susceptible animals from the poisonous action of tetanus toxin (within limits), but also against the effects of the living tetanus bacillus as well.

¹ See paper by Wassermann and Takaki, *Berliner klinische Wochenschrift*, 1898, No. 1, S. 5.

Tetanus antitoxin, though the first antitoxin discovered and frequently employed in the treatment of tetanus, has not yielded as brilliant results as those obtained with diphtheria antitoxin. There are good reasons why tetanus antitoxin may never be expected to yield such satisfactory results as does diphtheria antitoxin. Diphtheria infection can be recognized by bacteriological methods and the antitoxin administered long before very marked constitutional symptoms have developed, and consequently long before the diphtheria toxin has had time to bring about serious tissue alterations. In tetanus it is impossible to make such a definite bacteriological examination, and very frequently the first suggestion of the disease is the twitching of the muscles, the antecedent sign of the tetanic convulsions. When these clinical manifestations have developed in tetanus there is already very serious involvement of the central nervous system.

In the use of tetanus antitoxin it is advisable to employ it as early as possible and to give repeated doses until the symptoms are relieved. Whether the subdural administration of the antitoxin will be of greater value than the subcutaneous administration is as yet undecided.

A great deal of benefit results, from the administration of tetanus antitoxin as a prophylactic in the treatment of wounds in which infection by the tetanus bacillus is possible. The prophylactic injection of the tetanus antitoxin in these cases, however, should always be accompanied by approved surgical treatment of the wound, and under these conditions it is more or less doubtful which of these measures is of the greater value, but experience seems to indicate that the antitoxin has a distinct prophylactic influence in these cases.

BACILLUS EDEMATIS, LIBORIUS, 1886.

The bacillus of malignant edema, also known as *vibrion septicum*, is another pathogenic form almost everywhere present in the soil. In certain respects it is a little like *bacterium anthracis*, and was at one time confounded with it; but it differs in the marked peculiarity of being a strict anaërobe. It was first observed by Pasteur, but it was not until later that Koch, Liborius, Kitt, and others described its peculiarities in detail. It can often be obtained by inserting under the skin of rabbits or guinea-pigs small portions of garden-earth, street-dust, or decomposing organic substances. There results a widespread edema, with more or less gas-production in the tissues. In the edematous fluid about the site of inoculation the organism under consideration may be detected. (Fig. 102, A.)

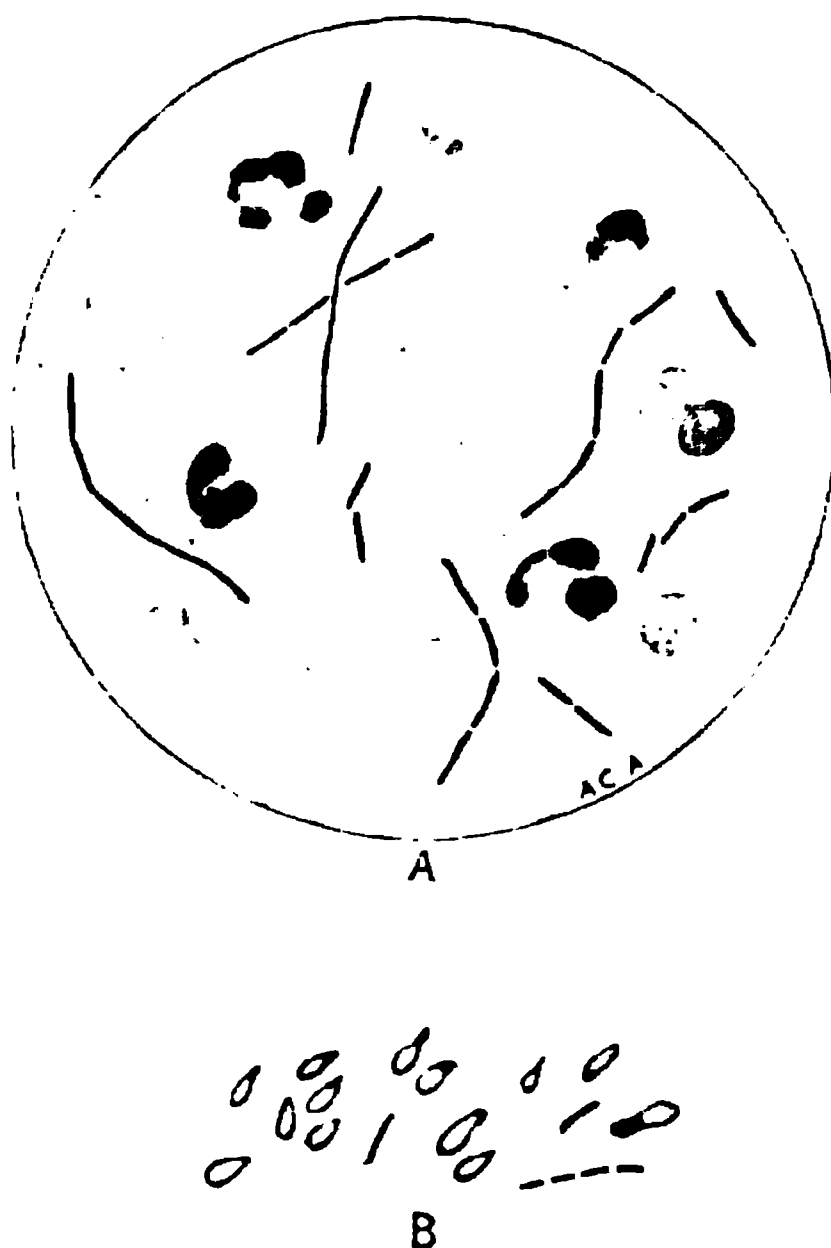
It is a rod about 3 to 3.5 μ long and from 1 to 1.1 μ thick—*i. e.*, it is about as long as *bacterium anthracis*, but is a trifle more slender. It is usually found in pairs, joined end to end, but may occur as longer threads; particularly is this the case in cultures. When in pairs the ends that approximate are squarely cut, while the distal extremities are rounded. When occurring singly both ends are rounded. (How does it differ in this respect from *bacterium anthracis*?) It is slowly motile, and its flagella are located both at the ends and along the sides of the rod. It forms spores that are usually located in or near the middle of the cells, causing frequently a swelling at the points at which they are located and giving to the cell a more or less oval, spindle, or lozenge shape. (Fig. 102, B.)

It is an obligate anaërobe, growing on all the ordinary

media, but not with access of oxygen. It grows well in an atmosphere of hydrogen. It causes liquefaction of gelatin.

In tubes containing about 20 to 30 c.c. of gelatin that has been liquefied, inoculated with a small amount of the

FIG. 102



Bacillus edematis. A, edema-fluid, from site of inoculation of guinea-pig, showing long and short threads; B, spore-formation, from culture.

culture, and then rapidly solidified in ice-water, growth appears in the form of isolated colonies at or near the bottom of the tube in from two to three days at 20° C. These colonies, when of from 0.5 to 1 mm. in diameter, appear as spheres filled with clear liquid, and are difficult, for this

reason, to detect. (Fig. 103.) As they gradually increase in size the contents of the spheres become cloudy and marked by fine radiating stripes, easily to be detected with the aid of a small hand-lens. In deep stab-cultures in agar-agar and in gelatin development occurs only along the track of puncture, at a distance below the surface. Growth is frequently accompanied by the production of gas-bubbles.

FIG. 103

It causes rapid liquefaction of blood-serum, with production of gas-bubbles, and in two or three days the entire medium may have become converted into a yellowish semifluid mass.

The most satisfactory results in the study of the colonies are obtained by the use of plates of nutrient agar-agar kept in a chamber in which all oxygen has been replaced by hydrogen. The colonies appear as dull whitish points, irregular in outline, and when viewed with a low-power lens are seen to be marked by a net-work of branching and interlacing lines that radiate in an irregular way from the centre toward the periphery.

It grows well at the ordinary temperature of the room, but reaches its highest development at the temperature of the body.

It stains readily with the ordinary aniline dyes. It does not stain by Gram's method.

Colonies of the bacillus of malignant edema in deep gelatin culture. (After Fränkel and Pfeiffer.)

Pathogenesis.—The animals known to be susceptible to inoculation with this organism are man, horses, calves, dogs, goats, sheep, pigs, chickens, pigeons, rabbits, guinea-pigs, and mice. Cases are recorded in which men and horses have developed the disease after injuries, doubtless due to the introduction into the wound, at the time, of soil or dust containing the organism.

If one introduce into a pocket beneath the skin of a susceptible animal about as much garden-earth as can be held upon the point of a penknife, the animal frequently dies in from twenty-four to forty-eight hours. The most conspicuous result found at autopsy is a wide-spread edema at and about the site of inoculation. The edematous fluid is in some places clear, while at others it may be stained with blood; it is usually rich in bacilli (Fig. 102, *A*) and contains gas-bubbles. Of the internal organs only the spleen shows much damage. It is large, dark in color, and contains numerous bacilli. If the autopsy be made immediately after death, bacilli are rarely found in the blood of the heart; but if deferred for several hours, the organisms will be found in this locality also, a fact that speaks for their multiplication in the body after death. At the moment of death they are present in varying numbers in all the internal viscera and on the serous surfaces of the organs.

Of all animals mice are probably the most susceptible to the action of this organism, and it is not rare to find it in the heart's blood, even immediately after death. They die, as a result of these inoculations, in from sixteen to twenty hours.

When a pure culture is used for inoculation a relatively large amount must be employed, and this should be deposited in the subcutaneous tissues at some distance from the surface.

In continuing the inoculations from animal to animal small portions of organs or a few drops of the edema-fluid should be used. The inoculation may also be successfully made by introducing into a pocket in the skin bits of sterilized thread or paper upon which cultures have been dried.

The methods for obtaining the organism in pure culture, from the cadaver of an animal that has succumbed to infection by the bacillus of malignant edema, are in all essential respects the same as those given for obtaining cultures from tissues in general; but it must be remembered that the organism is a strict anaërobe, and will not grow under the influence of oxygen. (See methods of cultivating anaërobic species.)

In certain superficial respects this bacillus suggests, as said above, *bacterium anthracis*, but differs from it in so many important details that there is no excuse for confounding the two.

NOTE.—From what has been said of this organism, what are the most important differential points between it and *bacillus anthracis*? Inoculate several mice with small portions of garden-earth and street-dust. Isolate the organism that agrees most nearly with the description here given for the bacillus of malignant edema. Compare its morphological, biological, and pathogenic peculiarities with those of *bacillus anthracis* under similar circumstances; especially its appearance in the tissues and fluids.

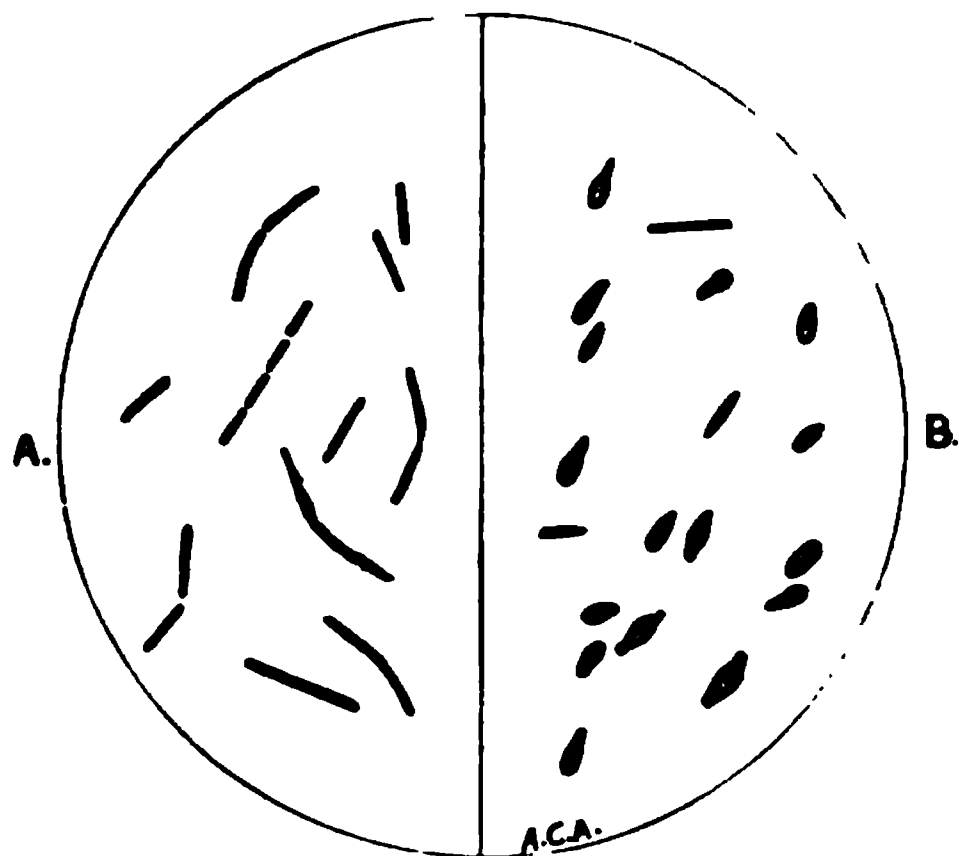
Still another pathogenic organism that may be present in the soil is

**BACILLUS CHAUVEI, ARLOING, CORNEVIN, AND
THOMAS, 1887.**

SYNONYMS: The bacillus of symptomatic anthrax—*Bactérie du charbon symptomatique* (Fr.)—*Bacillus des rauschbrand* (Ger.).

It is the organism concerned in the production of the disease of young cattle and sheep commonly known as "black leg," "quarter evil," and "quarter ill," a disease

FIG. 104



Bacillus of symptomatic anthrax. A, vegetative stage—gelatin culture; B, spore-forms—agar-agar culture.

that prevails in certain localities during the warm months, and which is characterized by a peculiar emphysematous swelling of the muscular and subcutaneous cellular tissues over the quarters. The muscles and cellular tissues at the points affected are seen on section to be saturated with bloody serum, and the muscles particularly are of a dark, almost black color. In these areas, in the bloody transu-

dates of the serous cavities, in the bile, and, after death, in the internal organs, the organism to be described can always be detected. It is manifest from this that the soil of localities over which infected herds are grazing may readily become contaminated through a variety of channels, and thus serve as a source of further dissemination of the disease.

FIG. 105

The organism was first observed by Feser, and subsequently by Bollinger and others. The most complete description of its morphological and biological peculiarities is that of Kitasato.¹ The following is from Kitasato's contributions: it is an actively motile rod about 3 to 5 μ long by 0.5 to 0.6 μ thick. It has rounded ends, and, as a rule, is seen singly, though now and then pairs joined end to end may occur. It has no tendency to form very long threads. (Fig. 104, A.)

It forms spores, and when in this stage is seen to be slightly swollen at or near one of its poles, the location in which the spore usually appears. (Fig. 104, B.) It is markedly prone to undergo degenerative changes, and involution-forms are commonly seen not only in fresh cultures, but in the tissues of affected animals as well.

Colonies of the bacillus of symptomatic anthrax in deep gelatin culture. (After Fränkel and Pfeiffer.)

¹ Zeitschrift für Hygiene. Bd. vi, S. 105; 13d. viii, S. 55.

Though actively motile when in the vegetative stage, it, like all other motile spore-forming bacilli, loses this property and becomes motionless when spores are forming.

It is strictly anaërobic and cannot be cultivated in an atmosphere in which free oxygen is present. It grows best under hydrogen, and does not grow under carbonic acid.

The media most favorable to its growth are those containing glucose (1.5 to 2 per cent.), glycerin (4 to 5 per cent.), or some other reducing-body, such as indigo-sodium sulphate, sodium formate, etc.

When cultivated upon gelatin plates in an atmosphere of hydrogen the colonies appear as irregular, slightly lobulated masses. After a short time liquefaction of the gelatin occurs and the colony presents a dark, dense, lobulated and broken centre, surrounded by a much more delicate, fringe-like zone.

When distributed through a deep layer of liquefied gelatin that is subsequently solidified colonies develop at only the lower portions of the tube. The single colonies appear as discrete globules that cause rapid liquefaction of the gelatin, and ultimately coalesce into irregular, lobulated liquid areas. In some of the larger colonies an ill-defined, concentric arrangement of alternate clear and cloudy zones can be made out. (Fig. 105.)

In deep stab-cultures in gelatin growth begins after about two or three days at 20° to 25° C. It begins usually at about one or two centimeters below the surface, and causes slow liquefaction at and around the track of its development. During its growth gas-bubbles are produced.

In deep stab-cultures in agar-agar at 37° to 38° C. growth begins in from twenty-four to forty-eight hours, also at about one or two centimeters below the surface, and is

accompanied by the production of gas-bubbles. There is produced at the same time a peculiar, penetrating odor somewhat suggestive of that of rancid butter. Under these conditions spores are formed after about thirty hours.

It grows well in bouillon of very slightly acid reaction under hydrogen, but does not retain its virulence for so long a time as when cultivated upon solid media. In this medium it develops in the form of white flocculi that sink ultimately to the bottom of the glass and leave the supernatant fluid quite clear. If the vessel be now gently shaken, these delicate flakes are distributed homogeneously through it. In bouillon cultures there is often seen a delicate ring of gas-bubbles round the point of contact of the tube and the surface of the bouillon. There is produced also a peculiar, penetrating, sour or rancid odor.

It grows best at the body-temperature—*i. e.*, from 37° to 38° C.—but can also be brought to development at from 16° to 18° C. Below 14° C. no growth is seen. Spore-formation appears much sooner at the higher than at the lower temperatures. When its spores are dried upon bits of thread in the desiccator over sulphuric acid, and then kept under ordinary conditions, they retain their vitality and virulence for many months. Similarly, bits of flesh from the affected areas of animals dead of this disease, when completely dried, are seen to retain for a long time the power of reproducing the disease. The spores are tolerably resistant to the influence of heat: when subjected to a temperature of 80° C. for one hour their virulence is not affected, but an exposure to 100° C. for five minutes destroys them. They are also seen to be somewhat resistant to the action of chemicals; when exposed to 5 per cent. carbolic acid they retain their disease-producing properties for about

ten hours, whereas the vegetative forms are destroyed in from three to five minutes; in corrosive sublimate solution of the strength of 1 : 1000 the spores are killed in two hours.

When gelatin cultures are examined microscopically the organisms are usually seen as single rods with rounded ends. When cultivated in agar-agar at a higher temperature spores are formed after a short time; the spores are oval, slightly flattened on their sides, thicker than the bacilli, and, as stated, frequently occupy a position inclining to one of the poles of the bacillus, though they are as often seen in the middle.

Bacilli containing spores are usually clubbed or spindle shape.

This bacillus stains readily with the ordinary aniline dyes. It is decolorized by Gram's method. Its spores may be stained by the methods usually employed in spore-staining.

Pathogenesis.—When susceptible animals, especially guinea-pigs, are inoculated in the deeper subcutaneous cellular tissues with pure cultures of this organism, or with bits of tissue from the affected area of another animal dead of the disease, death ensues in from one to two days. It is preceded by rise of temperature, loss of appetite, and general indisposition. The site of inoculation is swollen and painful, and drops of bloody serum may sometimes be seen exuding from it. At autopsy the subcutaneous cellular tissues and underlying muscles present a condition of emphysema and extreme edema. The edematous fluid is often blood-stained and the muscles are of a blackish or blackish-brown color. The lymphatic glands are markedly hyperemic. The internal viscera present but little alteration visible to the naked eye. In the blood-stained serous

fluid about the point of inoculation short bacilli are present in large numbers. These often present slight swellings at the middle or near the end. They are not seen as threads, but lie singly in the tissues. Occasionally two will be seen joined end to end. If the autopsy be made immediately after death, these organisms may not be detected in the internal organs; but if not made until after a few hours, they will be found there also. In recent autopsies only vegetative forms of the organism may be found; but later (in from twenty to twenty-four hours) spore-bearing rods may be detected. (How does this compare with *bacterium anthracis*?) By successive inoculations of susceptible animals with serous fluid from the site of inoculation of the dead animal the disease may be reproduced.

Cattle, sheep, goats, guinea-pigs, and mice are susceptible to infection with this organism, and present the conditions above described; whereas horses, asses, and white rats present only local swelling at the site of inoculation. Swine, dogs, cats, rabbits, ducks, chickens, and pigeons are, as a rule, naturally immune from the disease.

Though closely simulating the bacillus of malignant edema in many of its peculiarities, this organism can nevertheless, be readily distinguished from it. It is smaller; it does not develop into long threads in the tissues; it is more actively motile, and forms spores more readily in the tissues of the animal than does the bacillus of malignant edema. In their relation to animals they also differ; for instance, cattle, while conspicuously susceptible to symptomatic anthrax, are practically immune from malignant edema; and while swine, dogs, rabbits, chickens, and pigeons are readily infected with malignant edema, they are not, as a rule, susceptible to symptomatic anthrax. Horses

are affected only locally, and not seriously, by the bacillus of symptomatic anthrax; but they are conspicuously susceptible to both artificial inoculation and natural infection by the bacillus of malignant edema.

The distribution of the two organisms over the earth's surface is also quite different. The edema bacillus is present in almost all soils, while the bacillus of symptomatic anthrax appears to be confined to certain localities, especially places over which infected herds have been pastured.

A single attack of symptomatic anthrax, if not fatal, affords subsequent protection; while infection with the malignant edema bacillus appears to predispose to recurrence of the disease. (Baumgarten.)

BACTERIUM WELCHII, MIGULA, 1900.

SYNONYM: *Bacillus aërogenes capsulatus*, Welch and Nuttall, 1892.

This organism consists of straight or slightly curved rods with rounded ends, somewhat thicker than bacterium anthracis, varying in length from 3 to 6 μ ; sometimes longer chains or threads are seen. The rods are surrounded by a transparent capsule, whether grown in artificial media or obtained from animal bodies. It is a non-motile, spore-forming organism, and is strictly anaërobic in character. It stains with the ordinary aniline dyes and by the Gram method.

Under anaërobic conditions the organism grows on the usual culture media at room temperature, and forms large quantities of gas in media containing carbohydrates. Gelatin is not liquefied. In agar-agar the colonies are usually from 1 to 2 millimeters in diameter, but may be as large as 1 centimeter in diameter. They have a grayish-white color,

are flat, round or irregular masses, with small hair-like projections from the margin. In bouillon there is a diffuse clouding and marked white sediment. Milk is quickly coagulated. On potato there is a grayish-white layer.

The organism grows more rapidly at 30° to 37° C. than at 18° to 20° C. Cultures on agar-agar and bouillon have a slight odor resembling old lime. Bouillon cultures are killed after ten minutes at 58° C.

Bacterium Welchii was first described by Welch in 1891, and subsequently by Welch and Nuttall¹ in the blood and internal organs of a patient with thoracic aneurism opening externally. Autopsy was made eight hours after death and the vessels were found to contain large numbers of gas bubbles.

Injections of considerable quantities of cultures into the circulation of rabbits did not kill the animals, but if the animals were killed after being inoculated and were then allowed to lie at room temperature for twenty-four hours the organs and tissues were filled with gas bubbles.

Welch, Howard, Hitschman and Lilienthal, Hirschberg, and others have shown that the organism is frequently present in the feces of man and animals, as well as in the soil and in dust. Schattenfroh and Grassberger also found the organism in market milk.

BACILLUS SPOROGENES (KLEIN), MIGULA, 1900.

SYNONYM: *Bacillus enteritidis sporogenes*, Klein, 1895.

Klein found this organism in the intestinal discharges of infants and believed it had some relation to the acute inflammatory conditions of the intestinal tract of bottle-fed

¹ Bulletin Johns Hopkins Hospital, No. 24, 1892.

infants. The organism is very generally distributed in nature and can be very readily isolated from sewage by appropriate methods. It is an anaërobic, spore-forming organism, 0.8μ in width, and 1.6 to 4.8μ in length. It is actively motile and flagella have been demonstrated.

In culture media containing carbohydrates this organism produces gas in large quantities. Russell analyzed the gas and found it to be composed principally of methane. Milk and other sugar media in which the organism has been grown have a distinct odor of butyric acid.

When injected subcutaneously into guinea-pigs this organism causes most marked alterations. There is intense inflammation at the point of injection with edema and necrosis and the surrounding tissues are filled with gas. The bacteria are distributed throughout the body of the animal and can be isolated in pure culture from the blood of the heart. All the internal organs are intensely congested.

CHAPTER XXIX.

Bacteriological Study of Water—Methods Employed—Precautions to be Observed—Apparatus Employed, and Methods of Using It—Methods of Investigating Air and Soil—Bacteriological Study of Milk—Methods Employed.

BACTERIOLOGICAL STUDY OF WATER.

THE conditions that favor epidemic outbreaks of typhoid fever, Asiatic cholera, and other maladies of which these may be taken as types, have served as a subject for discussion by sanitarians for a long time.

Of the opinions that have been advanced in explanation of the existence and dissemination of these diseases, two should be considered: one, the ground-water doctrine of von Pettenkofer, because of its historic interest; the other, the belief that the diseases are disseminated by specifically polluted waters, because it is the view now prevalent among modern sanitarians.

The advocates of the "ground-water" view explained the occurrence of these diseases in epidemic form through alterations in the soil resulting from fluctuations in the level of the soil-water; and assigned to drinking-water either a very insignificant *rôle*, or ignored it entirely. On the other hand, those who have been instrumental in developing the drinking-water hypothesis claim that alterations in the soil play little or no part in favoring epidemic outbreaks; but that, as a rule, they appear as a result of direct infection, through the use of waters contaminated

with materials containing the specific organisms known to cause such diseases.

As a result of numerous observations by the disciples of both schools, the opinion is now general that polluted water is primarily the underlying cause of these epidemics, and this too, very often, when the state of the soil-water, in the light of the "ground-water" hypothesis, is just the reverse of what it should be in order to render it answerable for them. It is manifest, therefore, that the careful bacteriological study of water intended for domestic use is of the greatest importance, and should be a routine procedure in all communities receiving their water-supply from sources liable to pollution.

The object aimed at in such investigations should be to determine the number and kind of bacteria constantly present in the water—for all waters, except deep ground-water, contain bacteria; if sudden fluctuations in the number and kind of bacteria occur in these waters, and if so, to what they are due; and finally, and most important, whether the water contains constantly, or at irregular periods, bacteria that can be traced to human excrement, not of necessity pathogenic varieties, but bacteria that are known to be present normally in the intestinal canal. For if conditions are continuously favorable to pollution of the water by the normal constituents of the intestinal canal, the same conditions would allow of the occasional pollution of such water by infective matters from the *bowels* of persons suffering from specific disease of the intestines.

In considering water from a bacteriological standpoint it must always be borne in mind that comparisons with fixed standards are not of much value, for just as normal waters from different sources are seen to present variations

in their chemical composition, without being unfit for use, so may the relative number and variety of species of bacteria in water from one source be always greater or smaller than in that from another, and yet no difference may be seen to result from their employment. For this reason systematic study of any water, from this point of view, should begin with the establishment of what may be called its normal mean number of bacteria, as well as the character of the prevailing species; and in order to do this the investigations must cover a long period of time through all the seasonal variations of weather. From data obtained in this way it may be possible without analysis to predict approximately at any season the bacteriological condition of the water studied. Marked deviations from these "means," either in the quantity or quality of the organisms present, can then be considered as indicative of the existence of some unusual, disturbing element, the nature of which should be investigated. It is impossible to formulate an opinion of much value from either a single chemical or bacteriological analysis of a water, or from both together in many cases; for the results thus obtained indicate only the condition of the water at the time the sample was procured, and give no indication as to whether it differed at that time from its usual condition, or from the normal condition of the waters of the immediate neighborhood.

The interpretation of the results of both chemical and bacteriological analyses of a sample of water acquires its full value only through comparison, either with "means" that have been determined for this water, or with the results of simultaneous analyses of a number of samples from other sources of supply of the locality.

The aid of the bacteriologist is frequently sought in con-

nection with investigations of waters that are supposed to be concerned in the production of disease, particularly typhoid fever, either in isolated cases or in widespread epidemic outbreaks, and in these cases both the bacteriologist and the person employing his services are cautioned against being too sanguine of positive results, for in the vast majority of instances reliable bacteriologists fail to detect in these waters the bacillus that is the cause of typhoid fever.

Failure to find the organism of typhoid fever in water by the usual methods of analysis does not by any means prove that it is not present or has not been present. The means ordinarily employed in the work admit of such a very small volume of water being used in the test that we can readily understand how typhoid bacilli might be present in moderate numbers and yet none be included in the drop or two of the water taken for study. The conditions are not those of a *solution*, each drop of which contains exactly as much of the dissolved material as do all other drops of equal volume; but are rather those of a *suspension*, in every drop or volume of which the number of *suspended* particles is liable to the greatest degree of variation. Furthermore, there are other reasons that would, *a priori*, preclude our expecting to find the typhoid bacilli in water in which we may have reason to believe they had been deposited, because attention is not usually directed to the water until the disease has become conspicuous, usually in from two to four weeks after the pollution probably occurred. These intervals of time are ordinarily sufficient for the delicate, non-resistant bacillus of typhoid fever to succumb to the unfavorable conditions under which it finds itself in water. By unfavorable conditions are meant the absence of suitable nutrition; un-

favorable temperature; probably the antagonistic influence of more hardy saprophytic bacteria, particularly the so-called "water-bacteria," and of more highly organized water-plants; the effect of precipitation and of sedimentation; and, of great importance, the disinfecting action of direct sunlight.

Though the positive demonstration of typhoid bacilli in drinking-water by bacteriological methods is of extreme rarity, it must not be concluded that bacteriological analyses of suspicious waters shed no light upon the existence of pollution and the suitability or non-suitability of the water for drinking-purposes.

In the normal intestinal tract of human beings and domestic animals, as well as associated with the specific disease-producing bacillus in the intestines of typhoid-fever patients, is an organism that is frequently found in polluted drinking-waters, and whose presence is indicative of pollution by either normal or diseased intestinal contents; and though efforts may result in failure to detect the specific bacillus of typhoid-fever, the finding of the other organism, bacillus coli, justifies one in concluding that the water under consideration has been polluted by intestinal evacuations from either human beings or animals. Waters so exposed as to be liable to such pollution should never be considered as other than a continuous source of danger to those using them.

Another point to be remembered is in connection with chlorine as an indicator of contamination by human excrement. It is commonly taught that an excessive amount of chlorine in water points to contamination by human excreta. This may or may not be true, according to circumstances. A high proportion of this element in a sample

of water from a locality, the surrounding waters of which are poor in chlorine, is unquestionably a suspicious indication; but in a district close to the sea or near salt-deposits, for instance, where the proportion of chlorine (as chlorides) in the water is generally high, the value of the indications thus afforded is very much diminished unless the amount found in the sample under examination greatly exceeds the normal "mean," previously determined, for the amount of chlorine in the waters of the neighborhood.

A striking example of the latter condition occurred in the experience of the writer while inspecting a group of water-supplies on the east coast of Florida. In each instance the water was obtained from properly protected artesian wells, ranging from 200 to 400 feet deep, and located within a few hundred yards of the sea. The first sample subjected to chemical analysis revealed such an unusually high proportion of chlorine that, had this sample alone been considered, the opinion that it was polluted by human excreta might have been advanced. To prevent such an error samples of water from a number of wells in the neighborhood were examined, and they were all found to contain from ten to twelve times the amount of chlorine that ordinarily appears in inland waters, the excess being evidently due to leakage through the soil into the wells of water from the sea. In short, the presence of an excess of chlorine in water, while often indicating pollution from human evacuations, may nevertheless, sometimes arise from other sources; but the presence in water of bacteria normally found in the intestinal canal can manifestly admit of but one interpretation, viz., that fecal matters from either man or animals have at some time been deposited in this water, and that while no specific disease-producing organisms may

have been detected, still waters in which such pollutions are possible are a constant menace to the health of those who use them for domestic purposes.

A sudden variation from the normal, mean number of bacteria, or from the normal chemical composition of a water, calls at once for a thorough inspection of the supply, while at the same time the organisms present are to be subjected to the most careful study. In many instances, even after the most thorough bacteriological and chemical study of a suspicious water, one is forced to admit that information of but limited usefulness has been obtained through the employment of such analytical methods. In these cases too much stress cannot be laid upon the importance of a systematic inspection of the supply, and its relation to sources of pollution. Optical evidence of more or less dangerous contamination may often be obtained when laboratory methods fail to detect them. The reasons for such failure, in addition to those already given, are obvious—the polluting matters are often so diluted by the large mass of water into which they find their way as to be beyond recognition by the tests usually employed in such work, and still be present in amounts sufficient to originate disease.

The Qualitative Bacteriological Analysis of Water.—The qualitative bacteriological analysis of water entails much labor, as it requires not only that all the different species of organisms found in the water should be isolated, but that each representative should be subjected to systematic study, and its pathogenic or non-pathogenic properties determined.

For this purpose a knowledge of the methods for the isolation of individual species which have already been

described, and of the means of studying these species when isolated, is indispensable.

For this analysis certain precautions essential to accuracy are always to be observed.

The sample is to be collected under the most rigid precautions that will exclude organisms from sources other than that under consideration. If drawn from a spigot, it should never be collected until the water has been flowing for fifteen to twenty minutes in a full stream. If obtained from a stream or a spring, it should be collected, not from the surface, but rather from about one foot beneath the surface.

It should always be collected in vessels which have previously been thoroughly freed from all dirt and organic particles, and then sterilized; and the plates should be made as quickly as possible after collecting the sample.

When circumstances permit, all water analyses should be made on the spot where the sample is taken, as it is known that during transportation, unless the samples are kept packed in ice, a multiplication of the organisms contained in it always occurs.

For the purpose of qualitative analysis it is necessary that a small portion of the water—one, two, three, five drops—should first be employed for making the plates. In this way one can form an idea as to the approximate number of organisms in the water, and can, in consequence, determine the amount of water best suited for the plates. Duplicate plates are always to be made—one set upon agar-agar, which are to be kept in the incubator at body-temperature, and one set upon gelatin, to be kept at from 18° to 20° C.

As soon as colonies have developed the plates are to be

carefully compared and studied. It is to be noted if any difference in the appearance of the organisms on corresponding plates exists, and if so, to what it is due. It is to be particularly noted which plates contain the greater number of colonies, those kept at the higher or those at the lower temperature. In this way the temperature best suited for the growth of the majority of these organisms may be determined. As a rule, the greater number of colonies appear upon the gelatin plates kept at 18° to 20° C.; and from this it would seem that many of the normal water-bacteria do not find the higher temperature so favorable to their development as do the organisms not naturally present in water, particularly the pathogenic varieties. From these plates the different species are to be isolated in pure culture, the morphological and cultural characteristics determined, and finally, by tests upon animals, it is to be decided if any of them possess disease-producing properties.

NOTE.—What use should be made of this observation in examining water for the presence of pathogenic bacteria?

The waters most frequently studied from the qualitative bacteriological standpoint are those suspected of containing specific pathogenic bacteria—*i. e.*, waters polluted with sewage and with human excreta that are believed to be the source of infection of typhoid fever, or, less frequently, of Asiatic cholera. In the investigations of such water there are several points of which we should never lose sight, *viz.*, unless the water is under continuous study there is only a chance of detecting the specific pathogenic species, for, as a rule, the dangerous pollution occurs either but

once or is intermittent, so that even in the case of exposed streams there are periods when no specifically dangerous contamination may be in operation. As stated above attention is commonly called to the water when the disease, presumably caused by its use, is fully developed, and this is often days or weeks after the pollution of the stream really occurred. By an analysis made at this time one could scarcely hope to detect the specific organisms that had caused the disease, especially in water from flowing streams. The organisms sought for may have been present in the water and may have infected the users, and yet have disappeared by the time the sample taken for analysis was collected.

When present in polluted waters pathogenic bacteria are always vastly in the minority. Were they constantly present in large numbers infection among the users of such waters would be more frequent and more widespread than is commonly the case. They may be present in a water-supply in small numbers; they may even be in the sample supplied for analysis, and yet escape detection if only the ordinary direct plate method of isolation be used.

From these considerations it is obvious that before attempts are made to isolate the various species directly from a suspicious sample of water it is advisable to subject it to some method of treatment that will aid in separating the few specific pathogenic from the numerous common saprophytic species. For this purpose numerous methods have been devised. The most useful of these aim to favor the rapid multiplication of pathogenic forms that may be present and to suppress or check the growth of the ordinary water saprophytes.

Attention has been called to the fact that when exposed to the body-temperature many of the ordinary water-

bacteria develop only slowly or not at all, while under similar circumstances the disease-producing species develop most luxuriantly. Advantage has been taken of this observation in devising methods for this particular work, of which some of the following will prove serviceable:

Collect in a sterilized flask a sample of about 100 c.c. of the water to be tested, and add to this about 25 c.c. of sterilized bouillon of *four times the usual strength*. This is then placed in the incubator at 37° to 38° C., for thirty-six to forty-eight hours, after which plates are to be made from it in the usual way; the results will often be a pure culture of some single organism, either one of the intestinal variety or a closely allied species. By a method analogous to the latter the spirillum of Asiatic cholera has been isolated from water (see article on that organism); and by taking advantage of the effect of elevated temperature upon the bacteria of water Vaughan has succeeded in isolating from suspicious waters a group of organisms very closely allied to the bacillus of typhoid fever.

Theobald Smith has suggested a method by which it is easily possible to isolate, from waters in which they are present, certain organisms that are of the utmost importance in influencing our judgment upon the fitness of the water for domestic use. By the addition of small quantities—one, two, or three drops—of the suspicious water to fermentation-tubes (see article on Fermentation-tube) containing bouillon to which 2 per cent. of glucose has been added, and keeping them at the temperature of the body (37° to 38° C.), the growth of intestinal bacteria that may be present in the water is favored, while that of the water-organisms is not; in consequence, after from thirty-six to forty-eight hours the fermentation-characteristics of most

of these organisms is evidenced by the accumulation of gas in the closed end of the tube. From these tubes the growing bacteria can then be easily isolated by the plate method, and intestinal bacteria will not infrequently be found present.

For the isolation of the typhoid bacillus, especially from water, a host of other methods have been devised. Some of these aim, through the addition of special chemical reagents to the media, to retard the development of ordinary saprophytes without interrupting the growth of the colon and the typhoid bacillus. Most of these methods have proved disappointing. One of them, that of Parietti, still finds favor in the hands of some. It consists in adding to the culture-media to be used in the test varying amounts of the following mixture:

Phenol	5 grams
Hydrochloric acid	4 grams
Distilled water	100 c.c.

Of this solution 0.1, 0.2, and 0.3 c.c. are added respectively to each of three tubes containing 10 c.c. of nutritive bouillon. Several such sets of tubes are to be made. To each are then added from 1 to 3 c.c. of the water, and they are placed in the incubator at body-temperature. It is said that whatever development occurs consists only of the typhoid or colon bacillus, or both, if they were present in the original sample. They may then be isolated and separated by the usual plate method, or, better still, through the application of the methods of v. Drigalski and Conradi, of Ficker, or of Hoffmann and Ficker, or several of these methods in conjunction, detailed in the chapter on *bacillus typhosus*. Personally we have not had much success with the Parietti method. The typhoid bacillus has been isolated from water by passing very large quantities of water through an ordinary

Pasteur or Berkefeld filter, brushing off the matters collected on the filter into a sterilized vessel and examining this by plate methods.

It has occurred to us that possibly the employment of chemical coagulants, such as alum and iron, might prove serviceable for this purpose. Their action would be to mechanically drag down, in precipitating as hydroxides, the suspended bacteria contained in the fluid. This precipitate could then be examined bacteriologically, instead of the water, and the recent experiments of Ficker (*loc. cit.*) appear to demonstrate the value of such a procedure.

The difficulties in this field of work are obviously due to the suspension of a very small number of the disease-producing organisms sought for in large volumes of fluid, and the association with them of large numbers of other species that offer a very great obstacle to the successful search for the pathogenic varieties.

If by either of the above procedures bacilli that bear any resemblance to *bacillus typhosus* be isolated, recourse must then be had to all the differential tests detailed in the chapter on that organism.

The Quantitative Estimation of Bacteria in Water.—Quantitative analysis requires more care in the measurement of the exact volume of water employed, for the results are to be expressed in terms of the number of individual organisms to a definite volume. The necessity for making the plates at the place at which the sample is collected is to be particularly accentuated in this analysis, for multiplication of the organisms during transit is so great that the results of analyses made after the water has been in a vessel for a day or two are often very different from those that would have been obtained on the spot.

NOTE.—Inoculate a tube containing about ten cubic centimeters of sterilized distilled or tap water with a very small quantity of a solid culture of some one of the organisms with which you have been working, taking care that none of the culture-medium is introduced into the water-tube and that the bacteria are evenly distributed through it. Make plates at once from this tube, and on each succeeding day determine by counts whether there is an increase or diminution in the number of organisms—*i. e.*, if they are growing or dying. Represent the results graphically, and it will be noticed that in many cases there is during the first three or four days a multiplication, after which there is a rapid diminution; and, if the organism does not form spores, usually death in from ten to twelve days. This is not true for all organisms, but does hold for many.

Where it is not convenient, however, to make the analysis on the spot, the sample of water should be taken and packed in ice and kept on ice until the plates can be made from it, which should in all cases be as soon after its collection as possible.

For the collection of samples from the deeper portions of streams, lakes, etc., a number of convenient devices have been made. A very satisfactory apparatus has been made for me by Messrs. Charles Lentz & Sons, of Philadelphia. It consists of a metal frame-work, in which is encased a bottle provided with a ground-glass stopper. To the stopper a spring clamp is attached, and this in turn is operated by a string, so that when the weighted apparatus is allowed to sink into the stream the stopper may be removed from the bottle at any depth by simply pulling upon the string. When the bottle is filled with water the stopper is allowed to spring back into position by releasing the string. The

whole apparatus (depicted in Fig. 106) is provided with a weight that insures its sinking, and a heavy cord by which it may be lowered and raised. It should be sterilized before using. After collecting the sample the bottle should be wiped dry with a sterilized towel. Before removing the stopper the mouth of the bottle should be rinsed with alcohol and heated with a gas-flame, to prevent contamination of its contents by matters that may have been upon its surface.

FIG. 106

In beginning the quantitative analysis of water with which one is not acquainted certain preliminary steps are essential.

It is necessary to know approximately the number of organisms contained in any fixed volume, so as to determine the quantity of water to be employed for the plates or tubes. This is usually done by making preliminary plates from one drop, two drops, 0.25 c.c., 0.5 c.c., and 1 c.c. of the water. After each plate has been labelled with the amount of water used in making it, it is placed aside for development. When this has occurred one selects the plate upon which the colonies are only moderate in number—about 200 to 300 colonies presenting—and employs in the subsequent analysis the same amount of water that was used in making this plate.

Bottle for collecting water.

If the original water contained so many organisms that

there developed on a plate or tube made with one drop too many colonies to be easily counted, then the sample must be diluted with one, ten, twenty-five, fifty, or one hundred volumes, as the case may require, of sterilized distilled water. This dilution must be *accurate*, and its exact extent noted, so that subsequently the number of organisms per volume in the original water may be calculated.

The use of a drop is not sufficiently accurate. The dilution should therefore always be to a degree that will admit of the employment of a volume of water that may be exactly measured, 0.25 and 0.5 c.c. being the amounts most convenient for use.

Duplicate plates should always be made, and the mean of the number of colonies that develop upon them taken as the basis from which to calculate the number of organisms per volume in the original water.

For example: from a sample of water 0.25 c.c. is added to a tube of liquefied gelatin, carefully mixed and poured as a plate. When development occurs the number of colonies is too numerous to be accurately counted. One cubic centimeter of the original water is then to have added to it, under precautions that prevent contamination from without, 99 c.c. of sterilized distilled water—that is, we have now a dilution of 1 : 100. Again, 0.25 c.c. of this dilution is plated, and we find 180 colonies on the plate. Assuming that each colony develops from an individual bacterium, though this is perhaps not strictly true, we had 180 organisms in 0.25 c.c. of our 1 : 100 dilution; therefore in 0.25 c.c. of the original water we had $180 \times 100 = 18,000$ bacteria, which will be 72,000 bacteria per cubic centimeter (0.25 c.c. = 18,000, 1 c.c. = $18,000 \times 4 = 72,000$). The results are always to be expressed in terms of the number of bacteria per cubic centimeter of the original water.

Another point of very great importance (already mentioned) is the effect of temperature upon the number of colonies of bacteria that will develop on the plates made from water. It must always be remembered that a larger number of colonies appear on gelatin plates made from water and kept at 18° to 20° C. than on agar-agar plates kept in the incubator. The following table, illustrative of this point, gives the results of parallel analyses of the same waters, the one series of counts having been made upon gelatin plates at the ordinary temperature of the room, the other upon plates of agar-agar kept for the same length of time in the incubator at from 37° to 38° C. It will be seen from the table that much the larger number of colonies—*i. e.*, much higher results—were always obtained when gelatin was employed. The importance of this point in the quantitative bacteriological analysis of water is too apparent to require further comment.

TABLE COMPARING THE RESULTS OBTAINED BY THE USE OF GELATIN AT 18°-20° C. AND AGAR-AGAR AT 37°-38° C. IN QUANTITATIVE BACTERIOLOGICAL ANALYSES OF WATER. RESULTS RECORDED ARE THE NUMBER OF COLONIES THAT DEVELOPED FROM THE SAME AMOUNT OF VARIOUS WATERS IN EACH SERIES.¹

NUMBER OF COLONIES FROM WATER THAT DEVELOPED UPON—									
Gelatin plates at 18° to 20° C.					Agar-agar plates at 37° to 38° C.				
310	170
280	140
310	}	180
340		160
650	}	210
630		320
380	}	290
400		210
1000	}	100
890		130
340	}	280
370		210
490	}	110
580		100

¹ I am indebted to James Homer Wright, Thomas Scott Fellow in Hygiene 1892-1893), University of Pennsylvania, for the results presented in this table.

Another point of equal importance in its influence upon the number of colonies that develop is the reaction of the gelatin. A marked excess of either alkalinity or acidity always has a retarding effect upon many species found in water. Fuller's experience at the Lawrence (Mass.) Experiment Station has shown that gelatin of such a degree of acidity as to require the *further* addition of from 15 to 20 c.c. per litre of a normal caustic alkali solution to bring it to the phenolphthalein neutral point gives, on the whole, the best results. Thus, by way of illustration, Fuller found that a sample of Merrimac River water gave 5800 colonies per c.c. on phenolphthalein *neutral* gelatin, 15,000 colonies on gelatin that *would need* 20 c.c. of normal alkali solution to bring it up to the phenolphthalein neutral point—*i. e.*, a feebly acid nutrient gelatin, and 500 colonies on a gelatin so alkaline as to require 20 c.c. of a normal *acid* solution to bring it back to the phenolphthalein neutral point.

Throughout this part of the work it is to be borne in mind that when reference is made to plates it is not to a set, as in isolation experiments, but to a single plate.

Method of Counting the Colonies on Plates.—For convenience in counting colonies on plates or in tubes it is customary to divide the whole area of the gelatin occupied by colonies into smaller areas, and either count all the colonies in each of these areas and add the several sums together for the total, or to count the number of colonies in each of several areas, ten or twelve, take the mean of the results and multiply this by the number of areas containing colonies. The latter procedure obtains, of course, only when all the areas are of the same size. By this method, however, the results vary so much in different counts of *the same plate* that they cannot be considered as more than rough approximations.

NOTE.—Prepare a plate; calculate the number of colonies upon it by this latter method. Now repeat the calculation, making the average from another set of squares. Now actually count the entire number of colonies on the plate. Compare the results.

For facilitating the counting of colonies several very convenient devices exist.

Wolffhügel's Counting-apparatus.—This apparatus (Fig. 107) consists of a flat wooden stand, the centre of which is

FIG. 107

Wolffhügel's apparatus for counting colonies.

cut out in such a way that either a black or white glass plate may be placed in it. These form a background upon which the colonies may more easily be seen when the plate to be counted is placed upon it. When the gelatin plate containing the colonies has been placed upon this background of glass it is covered by a transparent glass plate which swings on a hinge. This plate, which is ruled in square centimeters and subdivisions, when in position is just above the colonies, without touching them. The gelatin

plate is moved about until it rests under the centre of the area occupied by the ruled lines. The number of colonies in each square centimeter is then counted, and the sum total of the colonies in all these areas gives the number of colonies on the plate; or, as has already been indicated, if the number of colonies be very great, a mean may be taken of the number in several (six or eight) squares; this is to be multiplied by the total number of squares occupied by the gelatin. The result is an approximation of the total number of colonies.

When the colonies are quite small, as is frequently the case, the counting may be rendered easier by the use of a small hand lens. (Fig. 108.)

FIG. 108



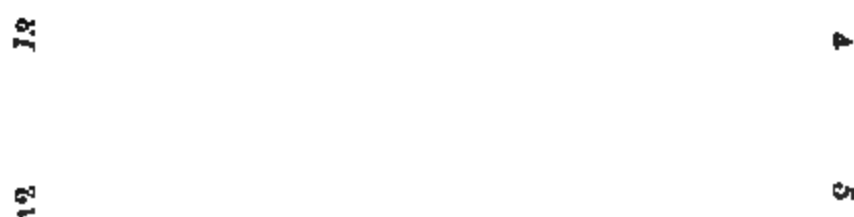
Lens for counting colonies.

Several useful modifications of the apparatus of Wolffhügel have been introduced. The most important is that of Lafar.¹ Lafar's counter consists of a glass disk of the diameter of ordinary size Petri dishes. It is supplied with a collar or flange that fits around the bottom of the Petri dish, and thus holds the counter in position. The disk is ruled with concentric circles, and its area is divided into sectors of such sizes that the spaces between the concentric circles and the radii forming the sectors are of equal size.

¹ *Centralblatt für Bakteriologie und Parasitenkunde*, 1891, Bd. xv, S. 331.

Three of the sectors are subdivided into smaller areas of equal size for convenience in counting when the colonies are very numerous. The principles involved are similar to those of the preceding apparatus, but the circular form of the apparatus admits of more exactness when counting colonies on a circular plate.¹

FIG. 109



Pakes' apparatus for counting colonies (reduced one-third).

Pakes² has introduced a cheap and convenient modification of Lafar's apparatus. It consists of a sheet of white paper on which is printed a black disk ruled with white lines, in somewhat the same fashion as is Lafar's counter,

¹ Lafar's apparatus is to be obtained from F. Mollenkopf, 10 Thorstrasse, Stuttgart, who holds the patent for it. Its price is about 8 marks.

² Journal of Bacteriology and Pathology, 1896, iv, No. 1.

though the areas of the smallest subdivisions are not of one size and do not bear a constant relation to each other.¹ To use this apparatus (Fig. 109) the Petri dish is placed centrally upon it, the cover of the dish is removed, and the colonies are counted as they lie over the spaces bounded by the white lines on the black disk beneath. When the plate is centered over the black disk the portion lying over one sector is exactly one-sixteenth of the whole plate.

FIG. 110

Esmarch's apparatus for counting colonies in rolled tubes.

Esmarch's Counter.—Esmarch devised a counter (Fig. 110) for estimating the number of colonies present upon a cylindrical surface, as when in rolled tubes. The principles and methods of estimation are practically the same as those given for Wolffhügel's apparatus.

¹ Copies of this apparatus are to be had of Ash & Co., 42 Southwark Street, London, or of Lentz & Sons, North Eleventh Street, Philadelphia, Pa. (The cost is but a few cents per copy.)

A simpler method than by the use of Esmarch's apparatus may be employed for counting the colonies in rolled tubes. It consists in dividing the tube by lines into four or six longitudinal areas, which are subdivided by transverse lines about 1 or 2 cm. apart. The lines may be drawn with pen and ink. They need not be exactly the same distance apart nor exactly straight. Beginning with one of these squares at one end of the tube, which may be marked with a cross, the tube is twisted with the fingers, always in one direction, and the exact number of colonies in each square as it appears in rotation is counted, care being taken not to count a square more than once; the sums are then added together, and the result gives the number of colonies in the tube. This method may be facilitated by the use of a hand-lens.

In all these methods there is one error difficult to eliminate: it is assumed that each colony has grown from a single organism. This is probably not always the case, as there may exist clumps of bacteria which represent hundreds or even thousands of individuals, but which still give rise to but a single colony—obviously this is of necessity estimated as a single organism in the water under analysis.

Where grounds exist for suspecting the presence of these clumps they may in part be broken up by shaking the original water with sterilized sand.

What has been said for the bacteriological examination of water holds good for all fluids which are to be subjected to this form of analysis.

The Sewage Streptococcus.—Houston¹ reached the conclusion that there is constantly present in sewage a particular form of streptococcus which is really more positively indica-

¹ Ann. Report, Local Gov. Board, xxviii.

tive of the contamination of water by sewage than is *bacillus coli*. This opinion was under investigation by members of the staff of the Massachusetts Institute of Technology, who reached the conclusion that considerable reliance can be placed upon the presence of this organism as an indication of sewage pollution of water.

The presence of the sewage streptococcus is most readily shown in the sediment in fermentation tubes inoculated with water under examination. If the sewage streptococcus is present it is very easy to demonstrate it by microscopic examination of the sediment after twenty-four to forty-eight hours. In addition to this test it has also been demonstrated by Winslow¹ that the estimation of the degree of acidity of the contents of the fermentation tube is a safe indication of the presence of the sewage streptococcus. When this organism is present the acidity rises far more rapidly and to a greater height than is the case when it is absent, so that in this way an additional indicator is available as to the potability of a water under examination.

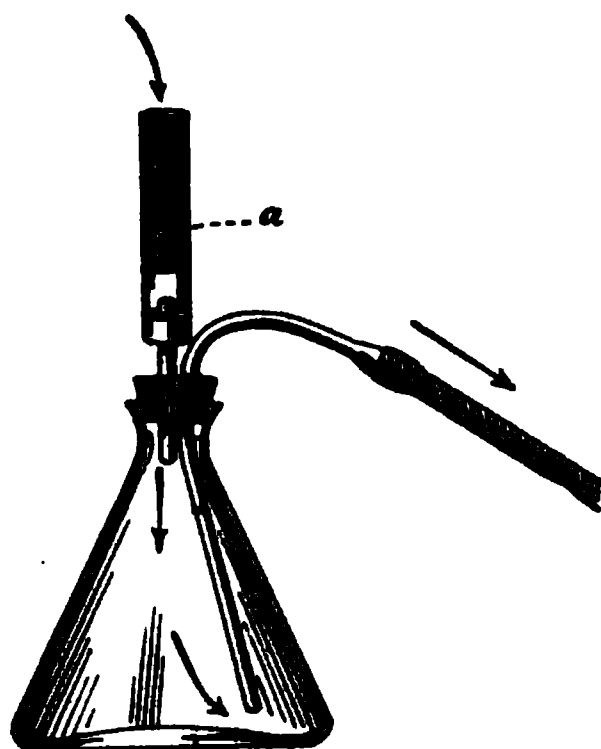
BACTERIOLOGICAL ANALYSIS OF AIR.

Quite a number of methods for the bacteriological study of the air exist. In the main they consist either in allowing air to pass over solid nutrient media (Koch, Hesse) and observing the colonies which develop upon the media, or in filtering the bacteria from the air by means of porous and liquid substances, and studying the organisms thus obtained. (Miguel, Petri, Strauss, Würz, Sedgwick-Tucker.) Because of their greater exactness, the latter have supplanted the former methods.

¹ Jour. Med. Research, 1902, vol. iii.

In some of the methods which provide for the filtration of bacteria from the air by means of liquid substances a measured volume of air is aspirated through liquefied gelatin; this is then rolled into an Esmarch tube and the number of colonies counted, just as is done in water analysis. This is the simplest procedure. An objection sometimes raised against it is that organisms may be lost, and not come into the calculation, by passing through the medium

FIG. 111



Petri's apparatus for bacteriological analysis of air. The tube packed with sand is seen at the point *a*.

in the centre of an air-bubble without being arrested by the fluid—an objection that appears to have more of speculative than of real value. Filtration through porous substances appears, on the whole, to give the best results. Petri recommends aspiration of a measured volume of air through glass tubes into which sterilized sand is packed. (Fig: 111.) When aspiration is finished the sand is mixed with liquefied gelatin, plates are made, and the number of

developing colonies counted, the results giving the number of organisms contained in the volume of air aspirated through the sand.

The main objection to this method is the possibility of mistaking a sand-granule for a colony. This objection has been overcome by Sedgwick and Tucker, who employ granulated sugar instead of sand; this, when brought into the liquefied gelatin, dissolves, and no such error as that possible in the Petri method can be made.

Sedgwick-Tucker Method.—On the whole, the method proposed by Sedgwick and Tucker gives such uniform results that it is to be preferred to others. It is as follows:

The apparatus employed by them consists essentially of three parts:

1. A glass tube of special form, to which the name *aërobioscope* has been given.
2. A stout copper cylinder of about sixteen litres capacity, provided with a vacuum-gauge.
3. An air-pump.

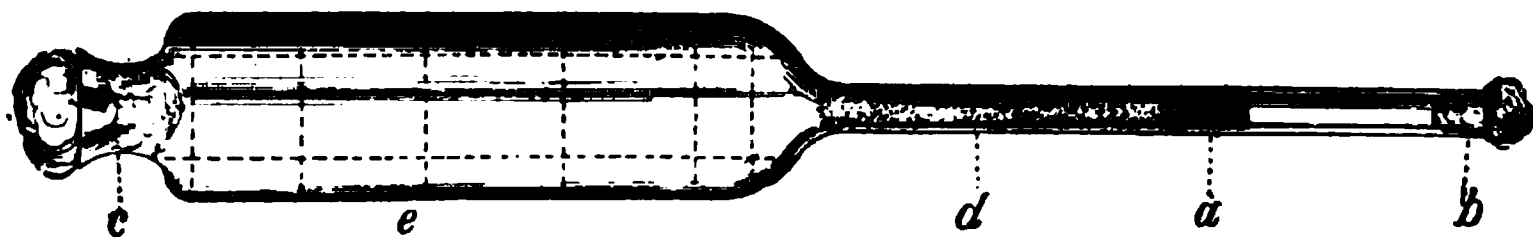
The *aërobioscope* (Fig. 112) is about 35 cm. in its entire length; it is 15 cm. long and 4.5 cm. in diameter at its expanded part; one end of the expanded part is narrowed to a neck 2.5 cm. in diameter and 2.5 cm. long. To the other end is fused a glass tube 15 cm. long and 0.5 cm. inside diameter, in which is to be placed the filtering-material.

Upon this narrow tube, 5 cm. from the lower end, a mark is made with a file, and up to this mark a small roll of brass-wire gauze (*a*) is inserted; this serves as a stop for the filtering-material which is to be placed over it. Beneath the gauze (at *b*), and also at the large end (*c*), the apparatus is plugged with cotton. When thoroughly cleaned, dried, and plugged, the apparatus is to be steril-

ized in the hot-air sterilizer. When cool the cotton plug is removed from the large end (*c*), and thoroughly dried and sterilized No. 50 granulated sugar is poured in until it just fills the 10 cm. (*d*) of the narrow tube above the wire gauze. This column of sugar is the filtering-material employed to engage and retain the bacteria. After pouring in the sugar the cotton-wool plug is replaced, and the tube is again sterilized at 120° C. for several hours.

Taking the air sample. In order to measure the amount of air used the value of each degree on the vacuum-gauge is determined in terms of air by means of an air-meter, or by calculation from the known capacity of the cylinder.

FIG. 112



The Sedgwick-Tucker aërobioscope.

This fact ascertained, the negative pressure indicated by the needle on exhausting the cylinder shows the volume of air which must pass into it in order to fill the vacuum. By means of the air-pump one exhausts the cylinder until the needle reaches the mark corresponding to the amount of air required.¹

A sterilized aërobioscope is now to be fixed in the upright position and its small end connected by a rubber tube

¹ Such a cylinder and air-pump are not necessary. A pair of ordinary aspirating bottles of known capacity graduated into litres and fractions thereof answer perfectly well. Or one can determine by the weight of water that has flowed from the aspirator the volume of air that has passed in to take its place—i. e., the volume of air that has passed through the aërobioscope.

with a stopcock on the cylinder, or to a glass tube tightly fixed in the neck of an aspirating-bottle by means of a perforated rubber stopper. The cotton plug is then moved from the upper end of the aërobioscope, and the desired amount of air is aspirated through the sugar. Dust-particles and bacteria will be held back by the sugar. During manipulation the cotton plug is to be protected from contamination.

FIG. 113

Bent funnel for use with aërobioscope.

When the required amount of air has been aspirated through the sugar the cotton plug is replaced, and by gently tapping the aërobioscope while held in an almost horizontal position the sugar, and with it, the bacteria, are brought into the large part (*e*) of the apparatus. When all the sugar is thus shaken down into this part of the apparatus about 20 c.c. of liquefied, sterilized gelatin is poured in through the opening at the end *c*, the sugar dissolves, and the whole

is then rolled on ice, just as is done in the preparation of an ordinary Esmarch tube.

The gelatin is most easily poured into the aërobioscope by the use of a small, sterilized, cylindrical funnel (Fig. 113), the stem of which is bent to an angle of about 110 degrees with the long axis of the body.

The larger part of the aërobioscope is divided into squares to facilitate the counting of the colonies.

By the employment of this apparatus one can filter the air at any place, and can then, without fear of contamination, carry the tubes to the laboratory and complete the analysis. Aside from this advantage, the filter being soluble only the insoluble bacteria are left imbedded in the gelatin.

For general use this method is to be preferred to the others that have been mentioned.

BACTERIOLOGICAL STUDY OF THE SOIL.

Bacteriological study of the soil may be made by either breaking up small particles of earth in liquefied media and making plates directly from this; or by what is perhaps a better method, as it gets rid of insoluble particles which may give rise to errors; breaking up the soil in sterilized water and then making plates immediately from the water.

It must be borne in mind that many of the ground-organisms belong to the anaërobic group, so that in these studies this point should be remembered and the methods for the cultivation of such organisms practised in connection with the ordinary methods. It must also be remembered that the nitrifying organisms, everywhere present in the ground, cannot be isolated by the ordinary methods, and will not

appear in plates made after either of the above plans. The special devices for their cultivation are described in the chapter on Soil-organisms.

BACTERIOLOGICAL STUDY OF MILK.

The possibility of milk serving as a vehicle in which disease-producing bacteria may be disseminated throughout a community has long been recognized, and epidemics of typhoid fever have been traced directly to infected milk, while such diseases as diphtheria and scarlet fever are also frequently regarded as being conveyed in the same manner.

In recent years the detailed study of the milk of individual cows has revealed the fact that streptococcus mastitis is not an uncommon occurrence in herds, and it has frequently been observed that milk rich in streptococci may prove dangerous when fed to infants and convalescents.

Since milk is such a favorable medium for the growth of a variety of bacteria it is not at all uncommon to find market milk very rich in bacteria, especially if it has been collected in a careless manner in dirty receptacles, in unsanitary stables, and has been shipped long distances at comparatively high temperatures.

For these various reasons the bacteriological study of milk has gained considerable prominence during the past few years—so much so that in some localities an effort is being made to establish a bacterial standard for market milk—that is, milk containing more than a certain number of bacteria is not regarded as suitable for use. Whether such a standard can be maintained or not remains to be demonstrated. The several milk commissions composed of pediatricists in various large cities have established a

bacterial standard for approved milk of 10,000 bacteria to the cubic centimeter. Experience has shown that it is possible to market milk that meets this bacterial standard sometimes with merely ordinary precautions with regard to cleanliness. In larger dairies it has frequently been a question of some difficulty on account of the elaborate scale on which the business is conducted.

Quantitative Bacteriological Analysis.—In the quantitative bacteriological examination of market milk it is necessary to dilute the milk with sterile water or sterile salt solution before plating on account of the very large numbers of bacteria present. The degree of dilution that is necessary will depend upon the nature of the dairy from which the milk is derived, the age of the milk, and the temperature at which it has been kept. Usually a dilution of 1 to 100, 1 to 1000, and 1 to 10,000 is sufficient. From these dilutions plate cultures are made with 0.1, 0.2, 0.3 cubic centimeter of each dilution.

Qualitative Bacteriological Analysis.—Aside from the quantitative bacteriological analysis of milk the qualitative analysis has received a great deal of attention. Detailed qualitative analysis necessarily entails an enormous amount of labor, but the detection of certain forms of bacteria is not always very difficult. This applies especially to the detection of streptococci.

Since milk containing streptococci in considerable numbers is derived from the udder of a cow suffering from some form of mastitis, it is always possible to find pus in such milk. Consequently it is customary to examine such milk for the presence of both streptococci and pus. This is done by centrifuging a cubic centimeter of the milk and collecting the sediment on a clean cover-slip and staining with Löffler's

methylene-blue. In this manner practically all the sediment derived from one cubic centimeter can be obtained on the cover-slip and a fairly satisfactory estimate can be made of the relative number of pus cells in this quantity of milk as well as at the same time an estimation of the relative number of streptococci.

Milk that shows pus cells along with distinct chains of streptococci, either extra- or intracellular, is usually regarded as dangerous in character, and boards of health usually direct that the cows from which such milk is derived be excluded from the dairy until such time as the milk is free from these elements.

APPENDIX.

LIST of apparatus and materials required in a beginner's bacteriological laboratory:

MICROSCOPE AND ACCESSORIES.

Microscope with coarse and fine adjustment and heavy, firm base; Abbe sub-stage condensing system, arranged either as the "simple" or as the regular Abbe condenser, in either case to be provided with iris diaphragm; objectives equivalent, in the English nomenclature, to about one-fourth inch and one-sixth inch dry, and one-twelfth inch oil-immersion system; a triple revolving nose-piece; three oculars, varying in magnifying power; and a bottle of immersion oil.

Glass slides, English shape and size and of colorless glass.

Six slides with depressions of about 1 cm. in diameter in centre.

Cover-slips, 15 by 15 mm. square and not more than from 0.15 to 0.18 mm. thick.

Forceps. One pair of fine-pointed forceps and one pair of the Cornet or Stewart pattern, for holding coverslips.

Platinum needles with glass handles. One straight, about 4 cm. long; one looped at the end, about 4 cm. long; and one straight, about 8 cm. long. Glass handles to be about 3 mm. in thickness and from 15 to 17 cm. long.

STAINING- AND MOUNTING-REAGENTS.

- 200 c.c. of saturated alcoholic solution of fuchsin.
- 200 c.c. of saturated alcoholic solution of gentian-violet.
- 200 c.c. of saturated alcoholic solution of methylene-blue.
- 200 grams of pure aniline.
- 200 grams of C. P. carbolic acid.
- 500 grams of C. P. nitric acid.
- 500 grams of C. P. sulphuric acid.
- 200 grams of C. P. glacial acetic acid.
- 1 liter of ordinary 93-95 per cent. alcohol.
- 1 liter of absolute alcohol.
- 500 grams of ether.
- 500 grams of pure xylol.
- 50 grams of Canada balsam dissolved in xylol.
- 100 grams of Schering's celloidin.
- 10 grams of iodine and 30 grams of potassium iodide in substance.
- 100 grams of tannic acid.
- 100 grams of ferrous sulphate.
- Distilled water.

FOR NUTRIENT MEDIA.

- $\frac{1}{4}$ pound of Liebig's or Armour's beef-extract.
- 250 grams of Witte's or Sargent's peptone.
- 2 kilograms of first quality gelatin.
- 100 grams of agar-agar in substance.
- 200 grams of sodium chloride (ordinary table-salt).
- 500 grams of pure glycerin.
- 50 grams of pure glucose.
- 20 grams of pure lactose.
- 100 grams of caustic potash.

200 c.c. of litmus tincture.
10 grams of rosolic acid (corallin).
Blue and red litmus-paper; curcuma paper.
5 grams of phenolphthalein in substance.
Filter-paper, the quality ordinarily used by druggists.
100 grams of pyrogalllic acid.
1 kilogram of C. P. granulated zinc.

GLASSWARE.

200 best quality test-tubes, slightly heavier than those used for chemical work, about 12 to 13 cm. long and 12 to 14 mm. inside diameter.

15 Petri double dishes about 8 or 9 cm. in diameter and from 1 to 1.5 cm. deep.

6 Florence flasks, Bohemian glass, 1000 c.c. capacity.

6 Florence flasks, Bohemian glass, 500 c.c. capacity.

12 Erlenmeyer flasks, Bohemian glass, 100 c.c. capacity.

1 graduated measuring-cylinder, 1000 c.c. capacity.

1 graduated measuring-cylinder, 100 c.c. capacity.

25 bottles, 125 c.c. capacity, narrow necks with ground-glass stoppers.

25 bottles, 125 c.c. capacity, wide mouths, with ground-glass stoppers.

1 anatomical or preserving jar, with tightly fitting cover, of about 4 liters capacity, for collecting blood-serum.

2 battery jars of about 2 liters capacity, provided with loosely fitting, weighted, wire-net covers for mice.

10 feet of soft-glass tubing, 2 or 3 mm. inside diameter.

20 feet of soft-glass tubing, 4 mm. inside diameter.

6 glass rods, 18 to 20 cm. long and 3 or 4 mm. in diameter.

6 pipettes of 1 c.c. each, divided into tenths.

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2 pipettes of 10 c.c. each, divided into cubic centimeters and fractions.

1 burette of 50 c.c. capacity, divided into cubic centimeters and fractions.

1 separating-funnel of 750 c.c. capacity for filling tubes.

2 glass funnels, best quality, about 15 cm. in diameter.

2 glass funnels, best quality, about 8 cm. in diameter.

2 glass funnels, best quality, about 4 or 5 cm. in diameter.

2 porcelain dishes, 200 c.c. capacity.

6 ordinary water tumblers for holding test-tubes.

1 ruled plate for counting colonies.

1 gas-generator, 600 c.c. capacity, pattern of Kipp or v. Wartha.

BURNERS, TUBING, ETC.

2 Bunsen burners, single flame.

1 Rose-burner.

1 Koch safety-burner, single flame.

6 feet of white-rubber gas-tubing.

12 feet of pure red-rubber tubing, 5 to 6 mm. inside diameter.

1 thermo-regulator, pattern of L. Meyer or Reichert.

2 thermometers, graduated in degrees of Centigrade, registering from 0° to 100° C., graduated on the stem.

1 thermometer graduated in tenths and registering from 0° to 50° C.

1 thermometer registering to 200° C.

INSTRUMENTS, ETC.

1 microtome, pattern of Schanze, with knife.

1 razor-strop.

- 6 cheap-quality scalpels, assorted sizes.
- 2 pair heavy dissecting-forceps.
- 1 pair medium-size straight scissors.
- 1 pair small-size straight scissors.
- 1 hypodermic syringe that will stand steam sterilization.
- 2 teasing-needles.
- 1 pair long-handled crucible-tongs for holding mice.
- 1 wire mouse-holder.
- 2 small pine boards on which to tack animals for autopsy.
- 2 covered stone jars for disinfectants and for receiving infected materials.

INCUBATORS AND STERILIZERS.

- 1 incubator, simple square form, either entirely of copper or of galvanized iron with copper bottom.
- 1 medium-size hot-air sterilizer with double walls, asbestos jacket, and movable false bottom of copper plates.
- 1 medium-size steam sterilizer; either the pattern of Koch or that known as the Arnold steam sterilizer, preferably the latter.

MISCELLANEOUS.

- 1 pair of balances, capacity 1 kilogram; accurate to 0.2 grams.
- 1 set of cork-borers.
- 1 hand-lens.
- 1 wooden filter-stand.
- 2 iron stands with rings and clamps.
- 3 round, galvanized iron-wire baskets to fit loosely into steam sterilizer.

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3 square, galvanized iron-wire baskets to fit loosely into hot-air sterilizer.

1 sheet-iron box for sterilizing pipettes, etc.

1 covered agate-ware saucepan, 1200 c.c. capacity.

2 iron tripods.

1 yard of moderately heavy wire gauze.

2 test-tube racks, each holding 24 tubes, 12 in a row.

1 constant-level, cast-iron water-bath.

2 potato-knives.

2 test-tube brushes with reed or wire handles.

Cotton-batting.

Copper wire, wire nippers.

Round and triangular files.

Labels.

Towels and sponges.

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